The Effect of Chronic Intermittent Hypoxia on Breast Cancer Cell Gene Expression and Malignant Properties

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Summary

Breast cancer is the most common cancer in women worldwide and the second most common cancer overall. The most aggressive and most lethal form of breast cancer is inflammatory breast cancer (IBC). The mechanisms underlying the aggressiveness of IBC are still poorly understood.

A key feature in the development and progression of breast cancer is the tumor microenvironment that is, amongst others, characterized by a specific composition of the extracellular matrix (ECM) and by the occurrence of tissue hypoxia and oxidative stress. Due to vascular remodeling, leading to structurally and functionally abnormal tumor vessels, tumor cells are exposed to alternating periods of hypoxia and reoxygenation, called chronic intermittent hypoxia (CIH). CIH is a cause of increased reactive oxygen species (ROS) production and has been identified as an important factor in promoting tumor progression and metastasis. However, the molecular mechanisms through which CIH increases the aggressiveness of breast cancer still need to be elucidated.

Here, we aimed to assess the impact of CIH on gene expression and cancer cell malignant properties in IBC and non-IBC cell lines. The triple-negative IBC cell line SUM149PT and the estrogen receptor-positive cell line T47D were exposed to 20 cycles of intermittent hypoxia (24 h 0.2% O_2, 48 h 21% O_2). The effects of CIH on the transcriptomic profile were analyzed in order to obtain insights into signaling pathways, which might be involved in the repeatedly reported enhancement of tumor aggressiveness upon CIH. CIH caused distinct changes in gene expression in both cell lines, with a much higher number of differentially expressed genes in SUM149PT cells compared to T47D cells. In T47D cells, we positively validated the differential gene expression of two ECM proteins which have been linked to tumor progression. In SUM149PT cells, CIH caused a strong upregulation of pro-metastatic genes encoding ECM proteins and inflammatory mediators. We positively validated the CIH-mediated increased mRNA and protein expression of the ECM protein tenascin-C (TNC), a key factor in tumor progression. Additionally, for the first time we identified an oxidative stress mediated regulation of TNC in IBC cells, which was dependent on activation of the NF-κB pathway but not on activation of other redox signaling pathways.

The observed changes in gene expression suggest an enhanced metastatic potential of SUM149PT cells. Performing *in vitro* assays to analyze the cancer cell malignant properties, we observed a trend towards a differential attachment behavior of SUM149PT cells following CIH.

In summary, this thesis identifies CIH in mediating tumor promotive gene expression changes. Furthermore, CIH and oxidative stress may play an important role in the constitutive activation of NF-κB in IBC, which has repeatedly been described in the literature.

Zusammenfassung

Brustkrebs ist die häufigste Krebserkrankung bei Frauen und die zweithäufigste insgesamt. Die aggressivste und letalste Brustkrebserkrankung ist das inflammatorische Mammakarzinom (IBC). Die der IBC Aggressivität zugrunde liegenden Ursachen sind weitgehend unbekannt.

Das Tumormikromilieu ist ein wichtiger Faktor in der Entwicklung und Progression von Brustkrebs. Es ist unter anderem durch eine bestimmte Zusammensetzung der extrazellulären Matrix (ECM) und durch das Auftreten von Gewebehypoxie und oxidativem Stress ausgezeichnet. Krebszellen sind alternierenden Phasen der Hypoxie und Reoxygenierung ausgesetzt, auch chronisch intermittierende Hypoxie (CIH) genannt. Diese resultiert aus dem Vorhandensein von strukturell und funktionell abnormalen Tumorgefässen und aus der Hypoxie-vermittelten Angiogenese. CIH generiert reaktive Sauerstoffspezies (ROS) und ist ein wichtiger Faktor, welcher die Tumorprogression und Metastasenbildung fördert. Die molekularen Mechanismen, durch welche CIH die Tumoraggressivität verstärkt, müssen noch aufgeklärt werden.

Das Ziel unserer Untersuchungen war es, den Effekt von CIH auf die Genexpression und die malignen Eigenschaften von IBC und nicht-IBC Zelllinien zu untersuchen. Die triple-negative IBC Zelllinie SUM149PT und die Estrogenrezeptor-positive Zelllinie T47D wurden für 20 Zyklen intermittierender Hypoxie (24 h 0.2% O_2, 48 h 21% O_2) ausgesetzt. Der Effekt von CIH auf die Genexpression wurde analysiert um eine Einsicht in die Signalwege zu bekommen, welche bei der wiederholt beobachteten Verstärkung der Tumoraggressivität durch CIH involviert sein könnten. CIH verursachte Zelllinien-spezifische Veränderungen der Genexpression, wobei SUM149PT Zellen eine höhere Anzahl verschieden regulierter Gene aufwiesen als T47D Zellen. Für T47D Zellen haben wir die differenzielle Genexpression zweier ECM Proteine, welche bekanntermassen mit der Tumorprogression assoziiert sind, positiv validiert. In SUM149PT Zellen verursachte CIH eine starke Aufregulierung pro-metastatischer Gene, welche für ECM Proteine und Entzündungsmediatoren kodieren. Wir validierten die CIH-vermittelten, erhöhten mRNA und Protein Werte des ECM Proteins Tenascin-C (TNC), einem entscheidenden Faktor in der Tumorprogression. Ausserdem zeigen wir als Erste eine durch oxidativen Stress vermittelte Regulierung von TNC in IBC Zellen, welche von der Aktivierung des NF-

κB Signalweges aber nicht von anderen Redox-regulierten Signalwegen abhängig war.

Die detektierten Veränderungen der Genexpression suggerieren ein erhöhtes metastatisches Potential der SUM149PT Zellen. Daher führten wir *in vitro* Experimente durch, um das Krebszellverhalten zu analysieren und fanden ein tendenziell verändertes Adhäsionsverhalten nach CIH.

Zusammengefasst identifiziert diese Dissertation CIH als Verursacher von tumorfördernden Veränderungen der Genexpression. Ausserdem könnten CIH und oxidativer Stress eine wichtige Rolle für die konstitutive NF-κB Aktivierung im IBC spielen, welche wiederholt in der Literatur beschrieben wurde.

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1. Introduction

1.1 The female breast

1.1.1 Anatomy of the female breast

The female breast is a bilateral organ that experiences large changes in shape, size and function during infantile growth, puberty, pregnancy, lactation and postmenopausal regression (J. Russo & Russo, 2004). The female breast hosts the mammary gland, a secretory organ that produces and secretes milk to feed infants. Alveoli are the basic components of a mature mammary gland, joining to groups to form lobules. Each lobule has a lactiferous duct draining into the nipple (Figure 1A+B). Epithelial cells of luminal and basal type form the ductal network of the gland. The inner luminal epithelium forms the ducts and secretory alveoli and has secretory or absorptive functions. The outer basal epithelium consists of myoepithelial cells (Watson & Khaled, 2008) which have contractile like properties that assist in milk ejection and support of the ductal-lobular system (Figure 1B) (Yoder, Wilkinson, & Massoll, 2007). The second predominant tissue in the female breast, next to the glandular tissue, is the adipose tissue (Figure 1A). Vascular endothelial cells form the blood vessels. Stromal cells, including fibroblasts, and a variety of immune cells are further located in the mammary gland (Watson & Khaled, 2008).

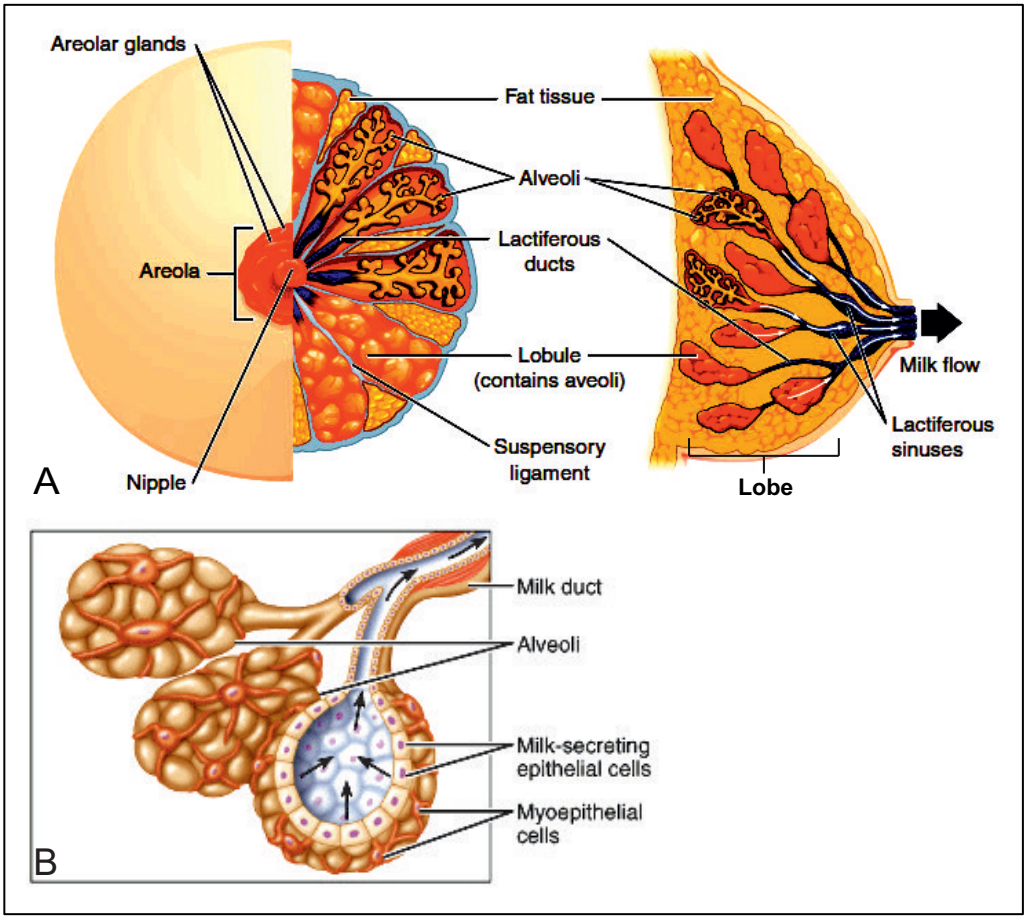

Figure 1: A) Anatomy of the mammary gland. Lobes (comprising numerous lobules) and ducts drain into the nipple and are embedded in the adipose tissue. **B)** Anatomy of a lobular-ductal unit. The basic units of lobules are alveoli consisting of milk-secreting epithelial cells and myoepithelial cells (adapted from http://themissionlsc.com/breast-anatomy.html).

1.1.2 Mammary gland development

The mammary gland development is initiated during the embryonic life. After birth, the mammary development halts until puberty. At puberty, growth occurs with the elongation of ducts and branching. The development and differentiation of the breast are only completed by the end of the first full term pregnancy (J. Russo & Russo, 2004). During pregnancy, tertiary branches form terminating in alveolar buds (Figure 2). Further, the luminal epithelium proliferates rapidly and differentiates to the secretory alveolar lineage. In the course of late pregnancy, a lactogenic switch occurs which is accompanied by the expression of the milk proteins, whey acidic protein (WAP) and α-lactalbumin, and by the formation of lipid droplets. Following weaning, removal of alveolar cells is accomplished by cell death. During post-lactational regression (involution), apoptosis leads to removal of about 80% of the epithelium within a few days (Watson & Khaled, 2008).

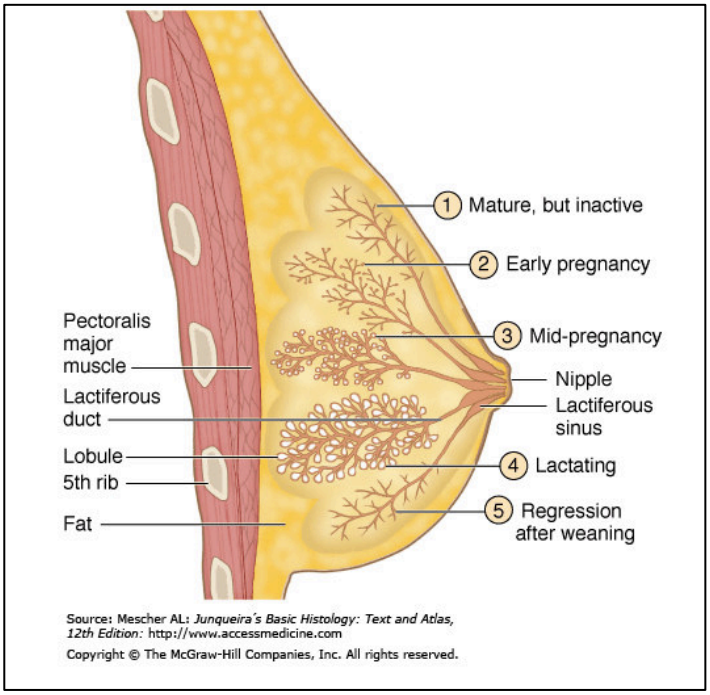

Figure 2: Sequence of changes occurring in the mammary gland during and after pregnancy and lactation. (1) Before pregnancy, ducts and alveoli are inactive. (2) In early pregnancy, branching occurs. (3) By mid-pregnancy, the secretory alveoli and ducts have dilated lumens. (4) At parturition and during the time of lactation, alveoli are greatly dilated and maximally active in production of milk components. (5) After weaning, alveoli and ducts regress with apoptotic cell death (Mescher, 2009).

1.2 Breast cancer

1.2.1 Incidence and risk factors

Breast cancer is the most common cancer in women worldwide and the second most common cancer overall (Ferlay et al., 2015). Its incidence continues to rise, while mortality is falling, partially resulting from earlier diagnosis and better therapies. Still, 20-30% of patients develop metastatic disease that remains incurable. Breast cancer metastases cause most of the cancer-related deaths among women (Torre et al., 2015).

The risk of breast cancer increases with the age at which a woman bears her first child (Rosner, Colditz, & Willett, 1994). An early first full term pregnancy appears to be protective, but the involved mechanisms are unknown. A full term pregnancy was postulated to inhibit carcinogenic initiation through the induction of differentiation, while an undifferentiated and highly proliferating mammary epithelium is a major target for carcinogenesis (I. H. Russo & Russo, 1996). This suggests that the breast of late parous and nulliparous women might have some of the undifferentiated and

cell proliferative characteristics that predispose the tissue to undergo neoplastic transformation (J. Russo & Russo, 2004). An additional factor that influences the risk of developing breast cancer is the diet. In general, obesity is a risk factor for postmenopausal breast cancer (Morimoto et al., 2002), which seems to be largely the result of an associated increase in estrogens, particularly bioavailable estradiol (Endogenous Hormones Breast Cancer Collaborative Group, 2003). Further, in premenopausal women, an increased risk of breast cancer was associated with high intake of animal proteins and red meat. On the other hand, high intake of polyunsaturated fatty acids, beta-carotene and soya proteins was associated with a decreased risk (H. P. Lee et al., 1991).

Hereditary factors are also involved in breast cancer susceptibility. *BRCA1/2*, *TP53*, *PTEN*, *LKB1* and *CDH1* are prominent genes whose mutations confer a high risk for developing breast cancer (Campeau, Foulkes, & Tischkowitz, 2008). Nevertheless, environmental factors were postulated to have a higher impact on the risk to develop breast cancer than genetic factors (Ziegler et al., 1993). Breast cancer incidence is higher in the United States than in China or Japan. However, when Chinese and Japanese women migrate to the United States, breast cancer risk rises over several generations and approaches that of U.S. whites (Ziegler et al., 1993).

Much research has been done in the fight against breast cancer but, despite huge discoveries and progress in the treatment of breast cancer, we still miss the complete understanding of the biologic heterogeneity of breast cancers regarding molecular alterations, treatment sensitivity and cellular composition. Hence, breast cancer still remains incurable in many cases.

1.2.2 Classification of breast cancer

Breast cancer is a complex disease with very distinct clinical, morphological and molecular entities. It is classified by histopathological type, grade, stage, receptor status and gene expression profiles.

1.2.2.1 Histopathological type

Current histological classification systems of breast neoplasms are based on tumor histology and the cell of origin. Breast sarcomas origin from the interlobular stroma while mixed fibroepithelial tumors origin from the intralobular stroma (Yoder et al., 2007). The majorities of breast cancer derive from the epithelium lining the ducts or

lobules and are either invasive or non-invasive (*in situ*). Ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS) are non-invasive, while invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) are invasive cancers (Peacock, 2001). A rare and very aggressive form of invasive breast cancer is inflammatory breast cancer (IBC) (F. M. Robertson et al., 2010).

Inflammatory breast cancer

IBC is the most aggressive form of breast cancer, with an incidence of up to 5% of all breast cancer cases in the United States (Anderson, Schairer, Chen, Hance, & Levine, 2005; Hance, Anderson, Devesa, Young, & Levine, 2005). The term "inflammatory" origins from its clinical presentation (Haagensen, 1971). IBC is characterized by erythema, edema, a "peau d'orange" appearance of the skin, breast enlargement, pain and tenderness (F. M. Robertson et al., 2010). IBC progresses very rapidly and has a lower overall survival compared to other breast cancers (van Uden, van Laarhoven, Westenberg, de Wilt, & Blanken-Peeters, 2015). In 50% of the patients, the cancer is not detectable as a palpable mass but rather diffusely distributed (W. T. Yang et al., 2008). Because of its very rapid onset of clinical signs and symptoms and its "inflammatory" appearance, IBC is often mistaken for a bacterial infection (Walshe & Swain, 2005). Almost all patients have lymph node metastases and 30% have distant metastases at the time of diagnosis (J. Li et al., 2011).

1.2.2.2 Grade

Grading determines the level of differentiation of cancer cells compared to normal breast tissue. Carcinomas are divided into low-grade (well differentiated), intermediate-grade (moderately differentiated) and high-grade (poorly differentiated), as cancer cells progressively loose the features of normal mammary cells (Dervan, 2001).

1.2.2.3 Stage

The classification into stages is based on the size of the tumor, whether or not the tumor has spread to the lymph nodes, and whether the tumor has metastasized. Larger size, nodal spread and metastasis have a larger stage number and a worse prognosis (Peacock, 2001).

1.2.2.4 Receptor status

Regarding the receptor status, protein levels of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) are determined in order to stratify patients for prognostic predictions and to select treatments (Khan et al., 2015).

1.2.2.5 Molecular subtype

Perou and colleagues were the first to classify breast cancer into intrinsic subtypes based on gene expression profiles. They defined four molecular subtypes: luminal, HER2, basal-like and normal breast (Perou et al., 2000). The subsequent expansion of this work established six breast cancer intrinsic subtypes: luminal A, luminal B, HER2-enriched, claudin-low, basal-like and a normal breast-like group (Prat et al., 2010; Sorlie et al., 2001). The intrinsic subtype classification provides the most valuable biological information on breast cancer. Figure 3 shows the intrinsic subtypes of breast cancer and their composition of by receptor status defined clinical groups.

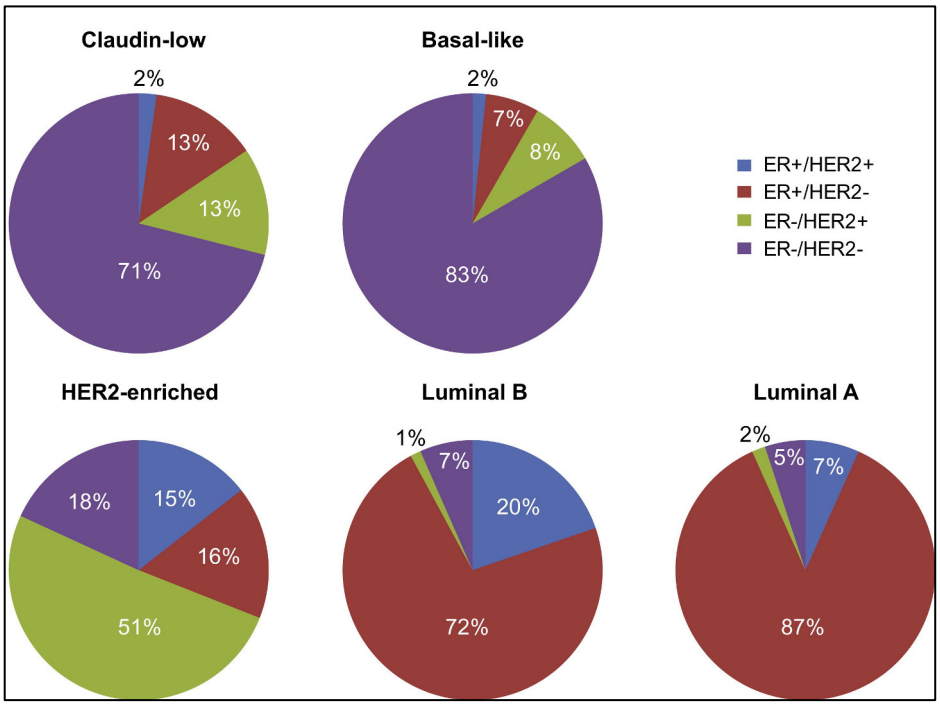

Figure 3: The intrinsic subtypes of breast cancer and their composition of clinical groups which are defined by the receptor status (Prat & Perou, 2011).

The intrinsic subtypes are characterized by distinct features (Table 1), such as expression levels of pro-proliferative genes, occurrence of specific gene mutations, histological tumor grade and prognosis (Figure 4).

Table 1: Features of intrinsic subtypes of breast cancer (adapted from (Eroles, Bosch, Alejandro Pérez-Fidalgo, & Lluch, 2012)).

Molecular Subtype	Frequency	Genes of Proliferation	Histologic grade	TP53 mutations	Prognosis
Basal-like	10-20%	High	High	High	Bad
Claudin-low	12-14%	High	High	High	Bad
HER2-enriched	10-15%	High	High	High	Bad
Luminal A	50-60%	Low	Low	Low	Excellent
Luminal B	10-20%	High	Intermediate/ High	Intermediate	Intermediate/ Bad
Normal breast-like	5-10%	Low	Low	Low	Intermediate

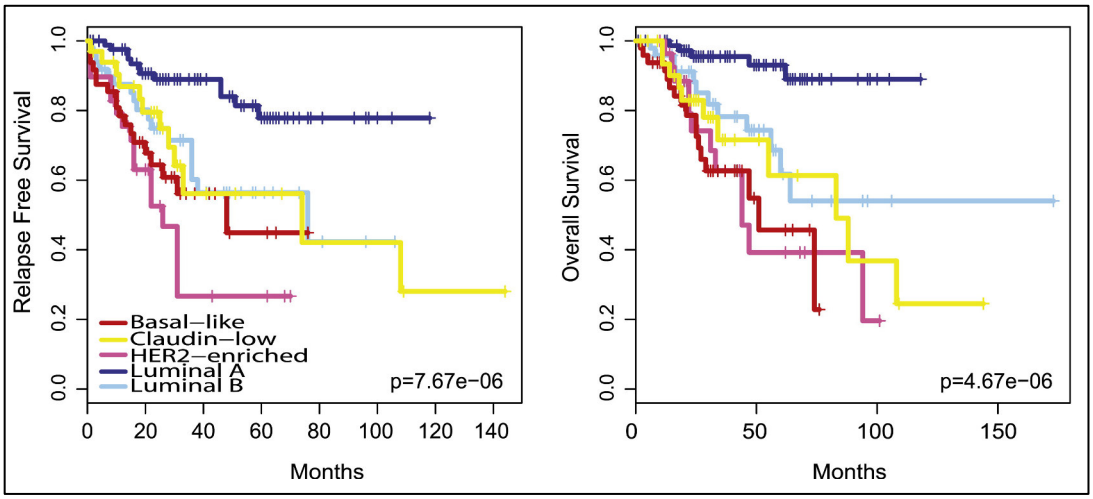

Figure 4: Kaplan-Meier relapse-free survival and overall survival curves based on UNC337 data set with normal breast-like samples excluded (Prat & Perou, 2011).

Luminal A

The luminal A breast cancer is with 50-60% the most common subtype. It is characterized by the expression of genes that are activated by the estrogen receptor (ER) transcription factor and typically expressed in the luminal epithelium lining the mammary ducts (Perou et al., 2000; Sorlie et al., 2001). Patients with this subtype of cancer have a good prognosis (Kennecke et al., 2010).

Luminal B

10-20% of all breast cancers are luminal B. They have a more aggressive phenotype, higher histological grade and proliferative index and a worse prognosis than luminal A-like breast cancers (Kennecke et al., 2010).

HER2-enriched

10-15% of all breast cancers are HER2-enriched. They show a high expression of the HER2 gene and other genes associated with the HER2 pathway and/or HER2 amplicon located in the 17q12 chromosome. These tumors are highly proliferative, 75% have a high histological grade and more than 40% have p53 mutations. The HER2 subtype is characterized by a poor prognosis (Parker et al., 2009; Prat & Perou, 2011).

Basal-like

10-20% of all breast carcinomas are basal-like. Tumors are of large size at diagnosis, show a high histological grade and a high frequency of lymph node affectation (Bosch, Eroles, Zaragoza, Viña, & Lluch, 2010). The most relevant feature is the absence of ER, PGR and HER2 expression. Therefore the terms basal-like and triple negative (TN) are often interchanged in clinical practice, even though the terms are not equivalent (Kreike et al., 2007). Basal-like tumors have a high rate of p53 (Sorlie et al., 2001) and BRCA1 mutations (Sørlie et al., 2003) which could explain their enormous aggressiveness and poor prognosis.

Claudin-low

12-14% of all breast cancers are of the claudin-low subtype. This type of breast cancer has its name because of the low expression of genes involved in tight junctions and intercellular adhesion, including claudin-3, -4, -7, cingulin, occludin, and E-cadherin (Parker et al., 2009; Prat et al., 2010). Claudin-low breast cancers overexpress genes closely linked to mesenchymal differentiation and epithelial-mesenchymal transition which is associated with the acquisition of a cancer stem cell (CSC) phenotype (Hennessy et al., 2009; Prat et al., 2010). Claudin-low tumors have a poor long-term prognosis (Prat et al., 2010).

Normal breast-like

5-10% of all breast carcinomas are of normal breast-like. Normal breast-like breast cancers are poorly characterized due to their rarity (Perou et al., 2000). There are doubts about their existence and some researchers believe them to be a technical artifact from high contamination with normal tissue during sample preparation for microarrays (Weigelt et al., 2010).

1.2.3 Breast cancer metastasis

Metastasis formation is the malignant tumor growth into secondary organs. Primary breast cancer cells metastasize to various distant organs, preferentially to the lung, liver and bone (Weigelt, Peterse, & van 't Veer, 2005). Metastasis formation involves invasion of carcinoma cells through the basement membrane into the stroma, followed by intravasation into lymphatic or blood vessels and systemic dissemination (Figure 5). Extravasation into secondary organs is followed by either dormancy or activation and colonization, which leads to the formation of a secondary tumor (Oskarsson, Batlle, & Massagué, 2014; Scheel & Weinberg, 2012). Basis for these processes is the epithelial-to-mesenchymal transition (EMT). EMT is a cellular program that confers the biological traits of mesenchymal cells to neoplastic epithelial cells. Typical differences between epithelial and mesenchymal cells are observed in their morphology, polarity, motility, cytoskeleton composition and specific cell-cell adhesion characteristics and result from differential transcription programs. One hallmark of EMT is the replacement of E-cadherin by N-cadherin. This results in the formation of weaker cell-cell adhesions between adjacent cells and is one feature that transmits migratory properties to previously immobile epithelial cells (Ye & Weinberg, 2015). At the last step of the metastatic cascade, it has been postulated that carcinoma cells switch back to an epithelial state through MET, thereby regaining their full proliferative phenotype (Chaffer et al., 2006; Spaderna et al., 2006).

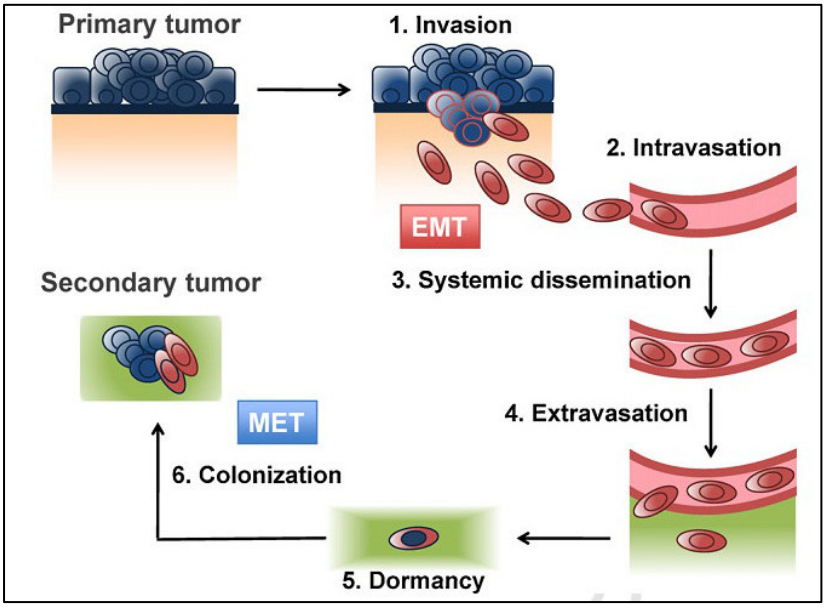

Figure 5: Scheme depicting the steps in the metastatic cascade. Carcinoma cells (blue) undergo EMT (changing to red), invade into the stroma (orange) and translocate to distant parenchyma (green). During colonization and secondary tumor formation, carcinoma cells are thought to switch back to an epithelial state through MET. EMT, epithelial-mesenchymal transition; MET, mesenchymal-epithelial transition (Scheel & Weinberg, 2012).

1.2.4 Treatment of breast cancer

The standard therapies in the treatment of breast cancer are surgery, radiation therapy and systemic therapy.

1.2.4.1 Surgery

One treatment option is the removal of cancerous and potentially affected breast tissue by surgery. A total mastectomy includes the total removal of the breast with the tumor. A breast-conserving mastectomy is the removal of the breast tumor with appropriate margins of the surrounding healthy tissue in a cosmetically acceptable manner (Franceschini et al., 2015).

1.2.4.2 Radiation therapy

Ionizing radiation is used to induce DNA damage and subsequently cellular death (L. Li et al., 2016). As cancer cells have a higher proliferation rate than non-cancerous cells, there is a higher likelihood that cancer cells are targeted by radiation therapy. Radiation therapy can be applied e.g. before mastectomy in order to shrink the tumor (neoadjuvant) or following a mastectomy (adjuvant).

1.2.4.3 Systemic therapy

In a systemic therapy, the treatment is taken orally or through intravenous injection to reach cancer cells throughout the body. The three general categories of systemic therapy used for breast cancer are chemotherapy, hormonal therapy and targeted therapy.

Chemotherapy

Chemotherapy is the administration of drugs targeting rapidly dividing cells by interfering with general cell division processes. The currently most common used chemotherapeutic agents are anthracyclines and taxanes (Bines, Earl, Buzaid, & Saad, 2014).

Hormonal therapy

The primary female sex hormones regulating diverse physiological processes are estrogens. Estrogen signaling is primarily mediated through estrogen receptors (ER) α and β, which act as ligand-activated transcription factors binding to estrogen response elements (EREs) located within the regulatory regions of target genes (Björnström & Sjöberg, 2005; Nilsson et al., 2001). Estrogens are also involved in pathophysiological processes, especially in the initiation and progression of breast cancer (Yager & Davidson, 2006). More than 50% of all breast cancers overexpress ERα and the presence of elevated ERα levels in the benign breast epithelium indicates an increased risk of breast cancer (Ali & Coombes, 2000). Most of ERα positive breast cancers depend on estrogen signaling for their growth and survival (Lappano et al., 2012). Hence, the use of ER antagonists and modulators is the primary treatment option for ER positive breast cancers. The most commonly used selective estrogen receptor modulator (SERM) is tamoxifen (Vogel et al., 2006).

Targeted therapy

Genetic alterations and transcriptional deregulations contribute to the initiation and development of breast cancer. Therefore, certain types of breast cancers are characterized by specific gene mutations or gene amplifications. Hence, novel cancer therapies target specific molecular defects that characterize certain cancer cells. This increases the treatment efficacy and reduces toxicities, as non-cancerous cells are not affected (Livraghi & Garber, 2015). The two main types of targeted therapies are

monoclonal antibody therapy and small-molecule therapy. HER2 positive tumors are treated with e.g. trastuzumab, an anti-HER2 monoclonal antibody, blocking the effect of overexpressed or amplified HER2 molecules (Gerber, 2008). Germline *BRCA1* and *BRCA2* mutations are one target in the treatment of *BRCA*-mutated breast cancers. Here, poly(ADP-ribose) polymerase (PARP) inhibitors are used to target *BRCA*-mutated cells following the principle of synthetic lethality (Livraghi & Garber, 2015).

1.3 The tumor microenvironment

A transformed cell that acquires the capabilities of autonomous proliferation, immortalization and invasion does not necessarily lead to the development of cancer. Moreover, transformed cells need to arise in an environment that supports the tumor development, called the tumor microenvironment (Bhome et al., 2015). The potential of cancer cells in forming metastases depends on their capability to facilitate favorable changes in the microenvironment of distant organs (Oskarsson et al., 2014). The tumor microenvironment includes the physicochemical and the cellular microenvironment, whereby the composition of the extracellular matrix (ECM) as well as tissue oxygenation and oxidative stress are important factors.

1.3.1 The extracellular matrix (ECM)

The ECM is a non-cellular three-dimensional macromolecular network composed of collagens, proteoglycans (PGs), glycosaminoglycans (GAGs), elastins, fibronectins, laminins and several other glycoproteins. Matrix components bind each other as well as cell adhesion receptors, constituting a complex network (Theocharis, Skandalis, Gialeli, & Karamanos, 2015). The ECM provides not only physical scaffolds, also cell surface receptors transduce signals from the ECM into the cells, regulating diverse cellular functions such as survival, growth, migration and differentiation (Theocharis et al., 2015). The ECM also serves as a reservoir for growth factors, cytokines and chemokines which can be liberated through cleavage from ECM components (Rozario & DeSimone, 2010).

1.3.1.1 The ECM and cancer

The extracellular matrix is a major player in cancer progression and metastasis (Hynes, 2009). During tumorigenesis, distinct alterations in the ECMs take place, leading to the formation of fibrotic stroma with increased stiffness, excessive deposition of ECM components and release of proteolytic enzymes which, upon activation, result in abnormal ECM remodeling (Theocharis et al., 2015). Proteolytic degradation of ECM components is mediated by enzymes, such as matrix metalloproteases (MMPs), a disintegrin and metalloproteases (ADAMs), ADAMs with thrombospondin motifs (ADAMTSs), cathepsins and plasminogen activators (Jarvelainen, Sainio, Koulu, Wight, & Penttinen, 2009).

Breast ducts and lobules are bilayered structures with an inner ring of luminal epithelial cells and an outer ring of myoepithelial cells. Myoepithelial cells are believed to play an important tumor suppressive role in the healthy breast as their presence inhibits invasion of cancer cells into the surrounding stroma (Gudjonsson et al., 2002; Ingthorsson, Hilmarsdottir, Kricker, Magnusson, & Gudjonsson, 2015). Myoepithelial cells surrounding tumors show a shift in ECM protein secretion, losing expression of tumor-suppressive laminins and increasing expression of collagens (Allinen et al., 2004; Ingthorsson et al., 2015). Surrounding the ducts and lobules is the breast stroma and stromal changes are observed even in the early stages of malignancy. This involves the appearance of cancer associated fibroblasts (CAFs), the recruitment of various immune cells, enhanced collagen I deposition and epigenetic modifications of stromal cells (Rudnick & Kuperwasser, 2012). A tumor promotive microenvironment is essential for the process of tumorigenesis (Figure 6) and an altered microenvironment can itself become a potent tumor promoter (Bissell & Hines, 2011).

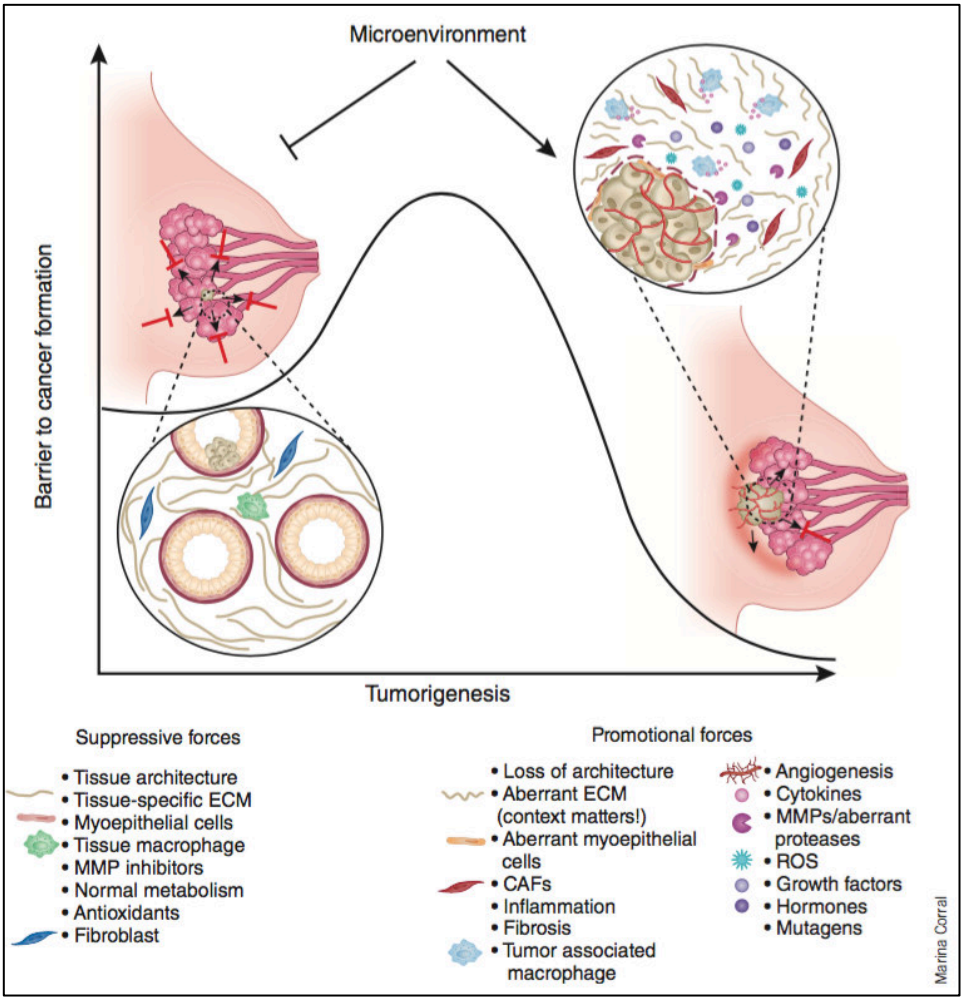

Figure 6: The tissue microenvironment can suppress or promote tumorigenesis (Bissell & Hines, 2011).

In breast cancers, high levels of fibronectin and its splice variants, crosslinked collagen I and tenascin-C are associated with poorer survival or time to progression for breast cancer patients. High levels of laminins, high molecular weight hyaluronic acid, heparins, versican, lumican or decorin correlate with better outcomes (C. Robertson, 2015). Next to changes in the expression levels of ECM components, changes in the spliceosome occur during the progression to malignancy (Venables, 2004). Here, ECM proteins with alternative splice forms are often observed to undergo isotype switching during the development of cancer (Di Modugno et al., 2012). Malignant cells express much higher levels of ED-A fibronectin and its receptor, $\alpha 5\beta 1$ integrin, both of which have been linked to radioresistance (Nam, Onodera, Bissell, & Park, 2010). Tenascin-C has also multiple alternative splice forms which are not observed in normal adult breast tissue (Adams, Jones, Walker, Pringle, & Bell, 2002). Furthermore an increased collagen deposition has been linked

to the progression of cancer, whereby invasive breast cancers showed an increased expression of fibrillar collagens compared to normal or to DCIS (Schummer et al., 2010). Fibrillar collagen I has been proposed as a link between increased mammographic breast density and increased risk of breast cancer (Martin & Boyd, 2008). Therefore, manual palpation is an important diagnostic method (Barton, Harris, Sw, JAMA 1999;282:1270-1280., & Nusbaum, 2001), whereby stiffness appears to increase with increasing tumor grade (Mullen, Thompson, Moussa, Vinnicombe, & Evans, 2014), concomitantly predicting a poorer prognosis (Berg et al., 2015). Successful chemotherapy has been shown to decrease tumor stiffness, whereas stiffening was observed in chemotherapy-resistant tumors (Falou et al., 2013).

1.3.2 Hypoxia and intermittent hypoxia

1.3.2.1 Occurrence and role in tumor progression

In healthy tissues, the oxygen supply matches the cellular requirements (normoxia). Hypoxia occurs when oxygen supply and consumption are not balanced and normal cellular functions cannot be maintained. The prevalence of hypoxic tissue areas is a feature of solid tumors, whereby hypoxic areas are heterogeneously distributed within the tumor mass (Vaupel & Mayer, 2007). Tumor hypoxia gives rise to a more malignant phenotype including the occurrence of metastases and it is associated with poor patient outcome in several types of cancer (Vaupel & Mayer, 2007).

Intratumoral hypoxia results from two major mechanisms. Tumor cells frequently outgrow their vascular supply territories caused by their abnormal growth patterns (Brown & Giaccia, 1998). This leads to a gradient of diffusion-limited oxygen supply (Figure 7), where some tumor cells are not sufficiently supplied with oxygen as they are in too far distance from the blood vessels (Denko, 2008).

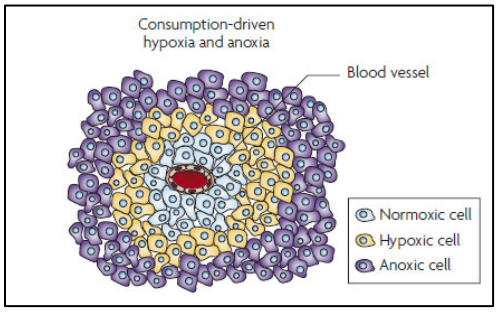

Figure 7: Diffusion-limited oxygen delivery in solid tumors (Denko, 2008).

Since hypoxia initiates angiogenesis, new blood vessels will grow into the hypoxic tumor microenvironment (Liao & Johnson, 2007). This enhances the oxygen supply and reduces the hypoxic areas until the tumor cells will outgrow this new supply again. However, the tumor neovasculature shows severe structural and functional abnormalities resulting in a perfusion-limited oxygen delivery that causes fluctuations of blood and hence oxygen supply (Figure 8) (Durand & Aquino-Parsons, 2001). Therefore, the tumor microenvironment is characterized by cycling periods of hypoxia and reoxygenation, also known as intermittent hypoxia (IH) (Dewhirst, Cao, & Moeller, 2008).

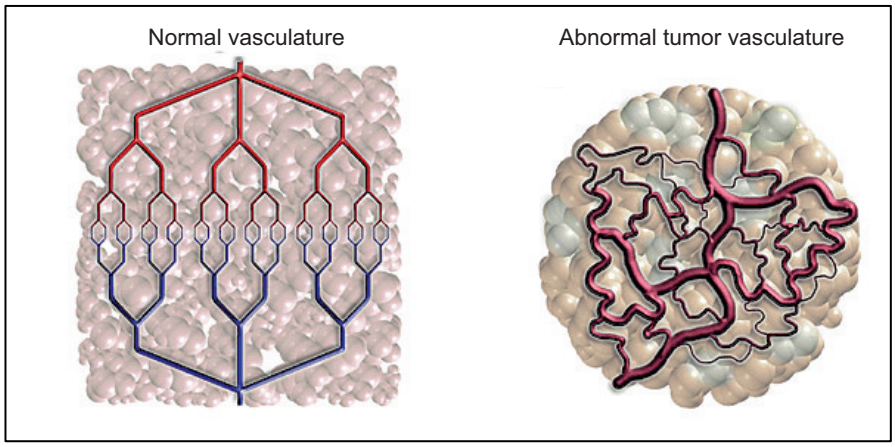

Figure 8: Comparison of normal vasculature and tumor vasculature. A disorganized tumor vasculature can cause a perfusion-limited oxygen delivery (Jain, 2001).

There are two dominant timescales that contribute to the cycling kinetics of IH. Vascular remodeling and angiogenesis are responsible for a slow frequency of cycling hypoxia (several cycles per day or week) (Bhaskara, Mohanam, Rao, & Mohanam, 2012; Cao et al., 2005). Variations in red blood cell flux within tumor microvessels can lead to a high frequency of hypoxic cycles (several cycles per hour) (Hsieh, 2010; Kimura et al., 1996).

Most past and current research aimed for the understanding of the role of chronic hypoxia in tumor progression and metastasis formation. However, the importance of IH is becoming more widely accepted and research on intermittent hypoxia increases. Chronic and intermittent hypoxia cause distinct effects, but IH has been shown to promote angiogenesis, resistance to anti-cancer treatment, intratumoral inflammation and tumor metastasis, to a higher extent than chronic hypoxia (Cairns,

Kalliomaki, & Hill, 2001; Chou et al., 2012; Hsieh, 2010; Kalliomäki, McCallum, Lunt, Wells, & Hill, 2008; Martinive et al., 2006; Tellier et al., 2015)

The molecular mechanisms causing these phenotypic changes are not well elucidated, but main factors might be changes in the regulation of transcription factors and changes in gene expression.

Cells adapt to the hypoxic microenvironment by up-regulating pro-survival mechanisms, the majority of which are coordinated by the transcription factor hypoxia inducible factor-1α (HIF-1α) (Maxwell, 2001). Further, the oscillating changes in tissue oxygenation are widely considered as a source of reactive oxygen species (ROS) (Dewhirst et al., 2008; Nanduri, Yuan, Kumar, Semenza, & Prabhakar, 2008). Several transcription factors have been reported to be activated by ROS. These are nuclear factor (erythroid-derived 2)-like 2 (Nrf2), activator protein-1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).

1.3.2.2 Hypoxia inducible factor (HIF)

Hypoxic conditions lead to the activation of the hypoxia-inducible factor (HIF) signaling pathway. The HIF transcription factors are heterodimers composed of an oxygen-sensitive regulatory α-subunit (HIF-α) and the constitutively expressed β-subunit (HIF-1β /ARNT (aryl hydrocarbon receptor nuclear translocator)). HIF α and -β proteins belong to the basic-helix-loop-helix (bHLH)/Per-ARNT-SIM (PAS) family of transcription factors. The bHLH domain mediates DNA-binding, while PAS and bHLH domains mediate heterodimerization with HIF-1β (Jiang, Rue, Wang, Roe, & Semenza, 1996; Semenza, 1999; G. L. Wang, Jiang, Rue, & Semenza, 1995). Three isoforms of the α-subunit existing in mammals, HIF-1α, HIF-2α and HIF-3α (Semenza, 2012). HIF-1α and HIF-2α share 48% amino acid sequence identity and contain amino- and carboxy-terminal oxygen-dependent degradation domains (NODDD and CODDD, respectively), and N-terminal and C-terminal transactivation domains (N-TAD and C-TAD). The domain structure of HIF-1α is shown in Figure 9. Beside a similar domain architecture, HIF-1α and HIF-2α are regulated in a comparable manner (O'Rourke, Tian, Ratcliffe, & Pugh, 1999; Schofield & Ratcliffe, 2004). HIF-3α is less closely related and its regulation is less well understood. Still, HIF-3α contains an ODDD, which includes the consensus oxygen-dependent prolyl hydroxylation motif, and can be targeted for ubiquitylation. Further, multiple alternatively splice variants have been reported (Maynard et al., 2003). One splice

variant is known as inhibitory PAS protein (IPAS). IPAS is composed of the amino-terminal basic helix-loop-helix and PAS domains, but omits the CODDD and other carboxy-terminal sequences. IPAS was shown to form transcriptionally inactive heterodimers with HIF-1α, leading to the inhibition of HIF-1α-dependent gene expression (Makino et al., 2001).

1.3.2.3 HIF signaling

The major mechanism for HIF regulation is mediated by enzymatic hydroxylation of specific residues in the NODDD, CODDD and C-TAD (Figure 9) (Schofield & Ratcliffe, 2004). In normoxia, HIF-α is continuously synthesized and rapidly degraded by the ubiquitin-proteasome system (L. E. Huang, Gu, Schau, & Bunn, 1998). 2-oxoglutarate and iron-dependent di-oxygenases, known as prolyl-4-hydroxylase domain (PHD)1, PHD2 and PHD3, hydroxylate HIF-α at two proline residues in the NODDD and CODDD (P564 and P402 in human HIF-1α, and P530 and P405 in human HIF-2α). These hydroxylations increase the affinity of the von Hippel-Lindau tumor suppressor protein (pVHL) for HIF-α (Ivan et al., 2001; Jaakkola et al., 2001). VHL forms part of a complex with elongins B and C, Cullin 2 and RING-Box protein 1 (RBX1) to constitute a functional E3 ubiquitin protein ligase. Subsequent polyubiquitination of HIF-α targets it for proteasomal degradation (Iwai et al., 1999). Additionally, the asparagine hydroxylase factor inhibiting HIF-1 (FIH-1) hydroxylates a specific asparagine residue within the C-TAD (N803 in human HIF-1α and N851 in human HIF-2α), preventing the recruitment of the transcriptional co-activators p300/CBP (p300 and CREB binding protein) and subsequent transactivation of target genes (Lando et al., 2002a; Lando, Peet, Whelan, Gorman, & Whitelaw, 2002b). In hypoxia, the activity of PHDs and FIH-1 is reduced, as they require molecular oxygen as a co-substrate (Jaakkola et al., 2001; Lando et al., 2002a), HIF-α protein accumulates, translocates into the nucleus and dimerizes with HIF-1β to form the transcription factor HIF. HIF binds DNA of target genes at hypoxia response elements (HREs), recruits p300/CBP and enhances transcription (Wenger, Stiehl, & Camenisch, 2005).

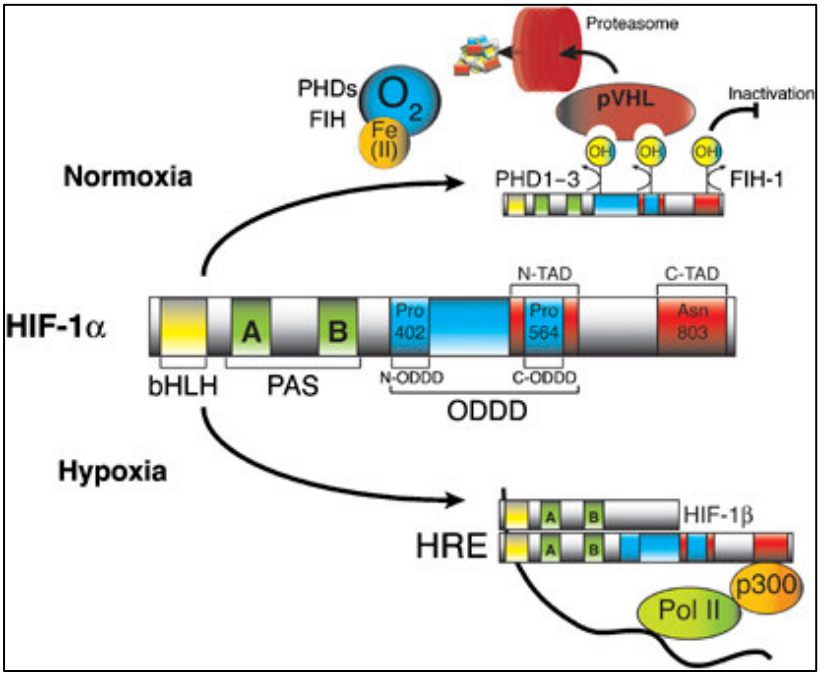

Figure 9: Domain structure of HIF-1α and its regulation in normoxia and hypoxia. In normoxia, PHDs hydroxylate HIF-1α, thereby targeting it for proteasomal degradation. FIH-1 mediated hydroxylation prevents cofactor recruitment. In hypoxia, hydroxylation is reduced, HIF-1α protein accumulates, translocates into the nucleus, dimerizes with HIF-1β and binds to HREs of target genes. PHD, prolyl-4-hydroxylase domain; FIH-1, factor inhibiting HIF-1; bHLH, basic-helix-loop-helix domain; PAS: Per/ARNT/SIM motif; ODDD: oxygen-dependent degradation domain; N-TAD: N-terminal transactivation domain; C-TAD: C-terminal transactivation domain; pVHL, von Hippel-Lindau protein; HRE: hypoxia response element (Weidemann & Johnson, 2008).

HIF-1α and HIF-2α have both overlapping and unique biological functions. HIF-1α induces gene expression of proteins involved in glycolysis (e.g. lactate dehydrogenase A (LDHA)) (Firth, Ebert, & Ratcliffe, 1995), while the control of erythropoiesis in vivo seems to be specific to HIF-2α (Rankin et al., 2007; Scortegagna et al., 2003), and modulation of angiogenesis is a common function of both HIF-1α and HIF-2α (e.g. vascular endothelial growth factor (VEGF)) (Hu, Wang, Chodosh, Keith, & Simon, 2003; Y. Liu, Cox, Morita, & Kourembanas, 1995). HIF-1α expression can be found in all nucleated cells, while HIF-2α expression seems to be restricted to specific cell types (Patel & Simon, 2008).

1.3.3 Reactive oxygen species (ROS), redox biology and oxidative stress

Reactive oxygen species (ROS) are by-products of aerobic metabolism. The most well studied ROS in cancer include superoxide anion ($O_2•^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (•OH) (Lambeth, 2004). ROS are required for various biological processes, thereby acting as secondary messengers. As a component of the cellular redox biology, small increases in ROS levels can activate signaling pathways, thereby initiating biological processes (Finkel, 2011). On the other hand, high levels of ROS can cause damage to lipids, proteins and DNA and are therefore described as oxidative stress. Oxidative stress has been suggested to induce pathologies like cancer (Cross et al., 1987). In order to counteract the detrimental effects of ROS cells produce antioxidant reducing cofactors and proteins like reduced glutathione (GSH), nicotinamide adenine dinucleotide phosphate (NADPH), thioredoxin (Trx), superoxide dismutases (SODs), catalase, glutathione peroxidases (GPxs) and peroxiredoxins (Prxs) (Cairns, Harris, & Mak, 2011; Holmström & Finkel, 2014).

Rapid proliferation of cancer cells causes high levels of ROS production. Additional mutations and adaptations, such as an increase of the antioxidant response, enable cancer cells to maintain ROS levels that allow pro-tumorigenic signaling pathways to be activated without inducing cell death (Cairns et al., 2011; Hayes & McMahon, 2009; Tai et al., 2012).

1.3.3.1 Cellular sources of ROS

There are many cellular sources of reactive oxygen species (ROS) within a cell. The electron transport chain in the mitochondria is the major source of ROS, through electron leakage from complex I and III (Figure 10A). The electrons react with molecular oxygen to produce superoxide. Further mitochondrial enzymes that are able to produce ROS are the pyruvate dehydrogenase (PDH), the 2-oxoglutarate dehydrogenase (OGDH), the electron transferring flavoprotein ubiquinone oxidoreductase (FQR), and the glycerol 3-phosphate dehydrogenase (GPDH) (Figure 10A) (Brand, 2010). Plasma membrane-bound NAD(P)H oxidases (NOXs) produce superoxide upon activation for a range of host defense and signaling functions (Figure 10B) (Morgan & Liu, 2011). Another source of ROS is the disulfide bond formation during protein folding in the endoplasmic reticulum (ER) (Figure 10C). Protein disulphide isomerase (PDI) accepts electrons from protein-folding substrates

and the electrons are transferred to molecular oxygen via ER oxidoreductin 1 (ERO1) (Tu & Weissman, 2002). Additional enzymes which can produce ROS are xanthine oxidase, cyclooxygenases, nitric oxide synthases, cytochrome P450 enzymes and lipoxygenases (Holmström & Finkel, 2014).

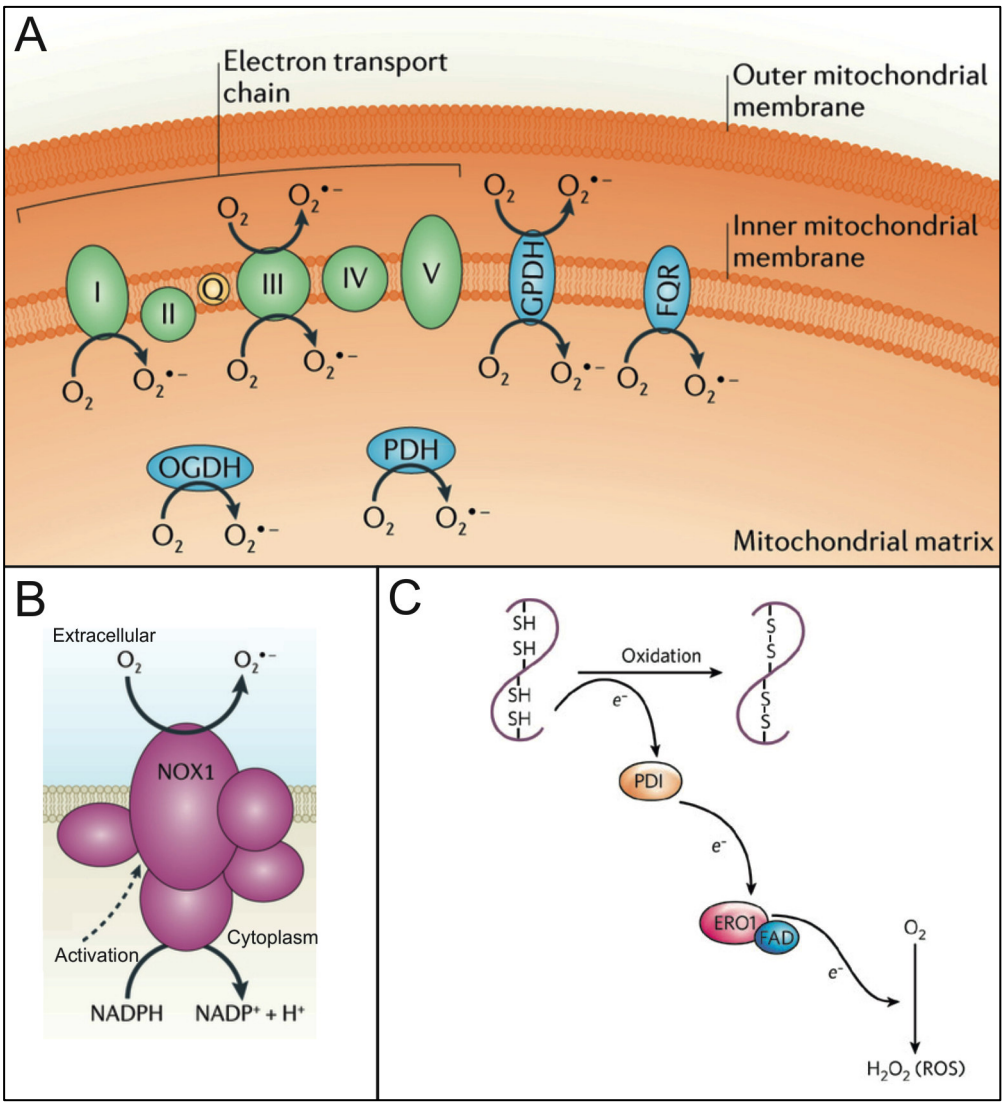

Figure 10: Cellular sources of ROS. **(A)** ROS are generated through electron leakage from complex I and III in the mitochondrial electron transport chain and through other mitochondrial enzymes. PDH, pyruvate dehydrogenase; OGDH, 2-oxoglutarate dehydrogenase; FQR, flavoprotein ubiquinone oxidoreductase; GPDH, glycerol 3-phosphate dehydrogenase (Holmström & Finkel, 2014). **(B)** Assembly and activation of the NADPH oxidase (NOX) superoxide-generating complex transfers electrons from cytosolic NADPH to molecular oxygen (adapted from Holmström & Finkel, 2014). **(C)** ROS are generated during protein folding. Protein disulphide isomerase (PDI) accepts electrons from protein-folding substrates and the electrons are transferred to molecular oxygen via ER oxidoreductin 1 (ERO1) (adapted from K. Zhang & Kaufman, 2008).

Generation of ROS during intermittent hypoxia

Several studies have reported an increased ROS production during oxygen deprivation followed by PHD inactivation and HIF-1α and HIF-2 α protein stabilization, whereby the mitochondrial electron transport chain was implicated as the major source of ROS (E. L. Bell et al., 2007; Duranteau, Chandel, Kulisz, Shao, & Schumacker, 1998; Guzy & Schumacker, 2006; Guzy et al., 2005). While an increased ROS production during hypoxia is highly debated, it has been widely accepted and studied that reoxygenation leads to ROS production (Littauer & de Groot, 1992). Reoxygenation-mediated ROS production was described in different *in vitro* studies, for example using U87 glioma cells or endothelial cells as cellular models (Hsieh, 2010; Toffoli et al., 2009). Moreover, reoxygenation has been shown to cause ROS-dependent DNA damage *in vitro* (Hammond, Dorie, & Giaccia, 2003). *In vivo*, reoxygenation that occurs after irradiation has been shown to produce ROS (Moeller, Cao, Li, & Dewhirst, 2004).

The acute reintroduction of oxygen during reoxygenation leads to a burst of ROS production that may result from different cellular sources, such as the mitochondrial electron transport chain (Holmström & Finkel, 2014). Another source may be the ER as unfolded proteins accumulate during hypoxia. Upon reoxygenation, disulfide bonds are rapidly introduced, leading to high level of ROS production (Koritzinsky & Wouters, 2013). Additionally, different NOX subunits have been shown to be inducible by ROS and Hsieh et al. demonstrated that the IH mediated increase of intracellular ROS production was dependent on NOX4 (Diebold et al., 2009; Djordjevic et al., 2005; Hsieh et al., 2012). Hence, elevated ROS levels following reoxygenation may induce the expression of NOX subunits thereby promoting a sustained ROS generation under these conditions. Furthermore, NOX2 and NOX4 have been shown to be HIF-1α target genes (Diebold, Petry, Hess, & Görlach, 2010; Diebold et al., 2012; Yuan et al., 2011). Hence, hypoxia may cause increased NOX2 and NOX4 protein levels, thereby even enhancing the NOX-mediated ROS production during reoxygenation.

1.3.3.2 Cysteine biochemistry and redox-dependent signaling

ROS can mediate changes in signal transduction by oxidative modification of proteins (Figure 11). Here, ROS oxidize the sulfhydryl group (SH) in redox-reactive cysteine residues resulting in a sulfenic form (-SOH), which can lead to the formation of intra-

or intermolecular disulfide bonds (-S-S-). Further oxidation of the sulfenic form results in sulfinic (-SO_2H) or sulfonic (-SO_3H) forms (Roos & Messens, 2011).

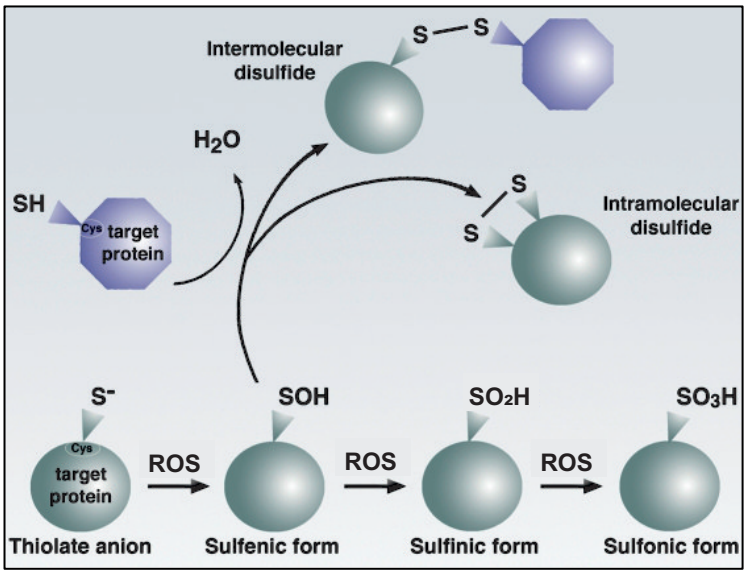

Figure 11: Mechanism of ROS mediated cysteine modification. ROS mediated oxidation of cysteine residues leads to the formation of the sulfenic form (-SOH), which can either form disulfide bonds or it is further oxidized to the sulfinic (-SO_2H) or sulfonic (-SO_3H) forms (adapted from Finkel, 2011).

1.3.3.3 Role of ROS in signal transduction by targeting phosphatases

ROS mediated oxidative modifications of phosphatases can cause changes in signal transduction. The oxidative modifications result in a differential structure and function of the protein. Thereby, protein tyrosine and dual-specificity phosphatases, like phosphatase and tensin homolog deleted on chromosome ten (PTEN) or mitogen-activated protein kinase (MAPK) phosphatases, can be inactivated and downstream signaling cascades may be activated (Kwon et al., 2004; S.-R. Lee et al., 2002; Salmeen et al., 2003; Seth & Rudolph, 2006).

The phosphatidylinositol 3 kinase (PI3K)/Akt pathway regulates a wide range of cellular processes including cell proliferation, migration and survival (Figure 12) (Cantrell, 2001). The tumor suppressor PTEN negatively regulates Akt activity by converting phosphatidylinositol-3,4,5-triphosphate (PIP_3) into phosphatidylinositol-4,5-bisphosphate (PIP_2) (Maehama, Taylor, & Dixon, 2001). *PTEN* is often mutated in many human cancers and loss of PTEN function has been shown to promote survival and proliferation of cancer cells (Cantrell, 2001; Katso et al., 2001). Also the ROS-mediated inactivation of PTEN can have pro-survival and pro-proliferative

effects by activation of the Akt pathway (Matsuda, 2013; F. Weinberg & Chandel, 2009).

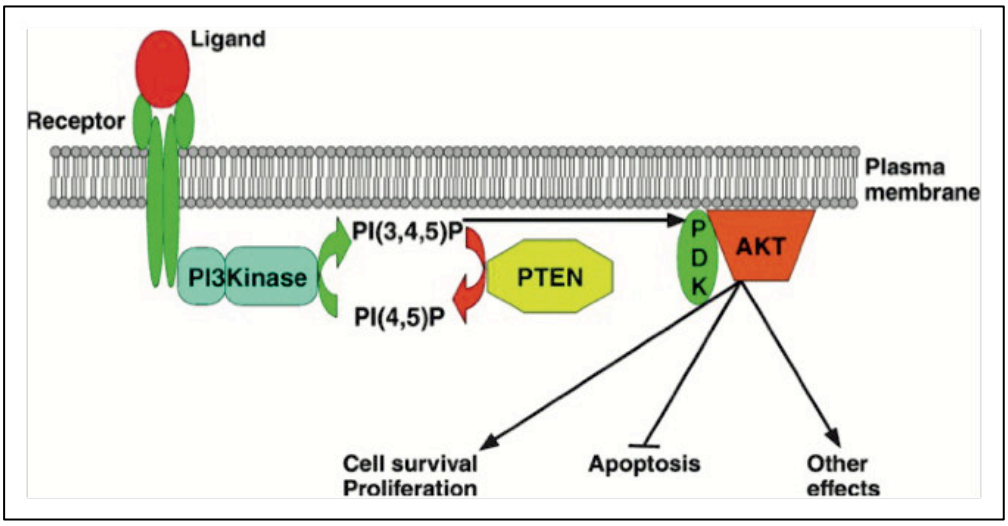

Figure 12: Model of the Akt pathway activation. Stimuli-dependent receptor activation leads to PI3K activation. Activated PI3K phosphorylates PI(4,5)P/PIP_2 to produce PI(3,4,5)P/PIP_3. PDK1 is recruited to the plasma membrane and activates Akt, which regulates various cellular processes, such as cell survival and proliferation. PTEN negatively regulates Akt activity by dephosphorylating PIP_3. PI3K, phosphatidylinositol 3 kinase; PI(4,5)P/PIP_2, phosphatidylinositol-4,5-bisphosphate; PI(3,4,5)P/PIP_3, phosphatidylinositol-3,4,5-triphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome ten; PDK1, 3-phosphoinositide-dependent protein kinase 1 (Planchon, Waite, & Eng, 2008).

Hence, elevated ROS levels in cancer cells can have tumor-promotive effects by activation of signaling pathway such as the Akt or MAPK pathways.

Oxidative modifications are reversible through reducing systems such as the thioredoxin system (Roos & Messens, 2011).

1.3.3.4 The thioredoxin system

An important antioxidant system, which is conserved in all species, is the thioredoxin system (Holmgren, 1985). The thioredoxin system includes the proteins thioredoxin and thioredoxin reductase. Thioredoxin contains an active site motif of Cys-Gly-Pro-Cys, and exists either in a reduced form (thioredoxin-(SH)2) with a dithiol, or in an oxidized form (thioredoxin-S2) by formation of an intramolecular disulfide bridge (Holmgren, 1985). Reduced thioredoxin catalyzes the reduction of disulfide bonds in other oxidized proteins. In this process thioredoxin becomes oxidized and a disulfide bond forms between the two cysteine residues in its active site (Figure 13).

Thioredoxin reductase restores thioredoxin to a reduced state with the use of NADPH (Holmgren, 1985). Thioredoxin can also directly scavenge reactive oxygen species (K. C. Das & Das, 2000) and it further regulates the activity of other important enzymes, such as peroxiredoxins (Rhee, Chae, & Kim, 2005), which also maintain the cellular redox balance. Peroxiredoxins use their SH groups to reduce peroxides such as H_2O_2, organic hydroperoxides and peroxynitrite (Figure 13). The oxidized form of peroxiredoxins is then reversed to the active reduced form by thioredoxin (Rhee et al., 2005).

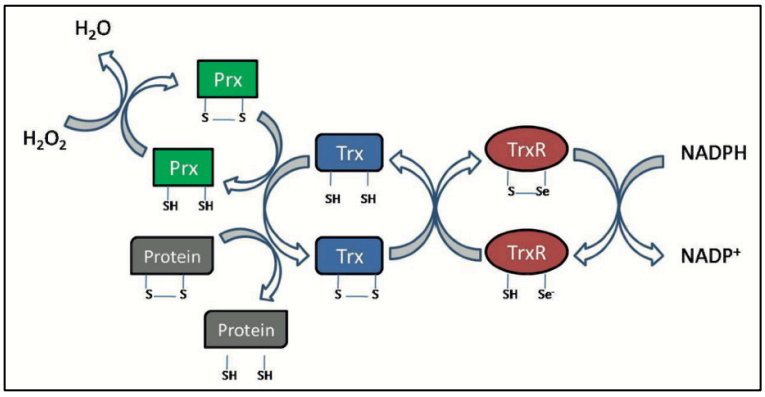

Figure 13: Mechanisms of the thioredoxin (Trx) system. Reduced Trx mediates the reduction of disulfides (S-S) within oxidized cellular proteins, like peroxiredoxin (Prx). Trx becomes oxidized, which in return is reduced by thioredoxin reductase (TrxR) at the expense of NADPH (adapted from Karlenius & Tonissen, 2010).

1.3.3.5 MAPK/AP-1 signaling and its regulation by ROS

The mitogen-activated protein kinase (MAPK) signaling pathways harbor four major MAPKs: the extracellular signal-related kinases (Erk1/2), the c-Jun N-terminal kinases (JNK), the p38 kinase (p38), and the big MAP kinase 1 (BMK1/Erk5). Upon stimulation by growth factors, hormones, cytokines and also genotoxic and oxidative stress, MAPK kinase kinases (MAPKKK) phosphorylate and activate MAPK kinases (MAPKK), which in turn phosphorylate and activate MAPKs (Morrison, 2012).

Activator protein 1 (AP-1) is a transcription factor involved in MAPK signaling that has been shown to be regulated by ROS (Chang & Karin, 2001). AP-1 regulates many cellular processes, including cell proliferation, death, survival and differentiation. AP-1 is a dimer composed of basic-region-leucine zipper (bZIP) proteins that belong to the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1, Fra-2), Maf (c-Maf, MafB, MafA, MafG/F/K, Nr1) and ATF (ATF2, LRF1/ATF3, B-ATF, JDP1, JDP2) sub-families (Shaulian & Karin, 2002). The differential AP-1 dimer composition alters its

DNA-binding specificity and determines its molecular function and thus the regulation of specific target genes. c-Jun is the most potent transcriptional activator in its group, forming stable heterodimers with e.g. Fos proteins (Shaulian & Karin, 2002). During tumorigenesis, c-Jun is known as an essential regulator of major cellular processes, such as cell proliferation. Jun-Fos dimers were shown to promote proliferation and neoplastic transformation (van Dam & Castellazzi, 2001). c-Jun overexpressing cells demonstrated higher mobility and invasiveness and loss of polarity in mammary epithelial cells (Bos, Margiotta, Bush, & Wasilenko, 1999; Fialka, 1996).

The regulation of AP-1 activity is complex and occurs at different levels (Shaulian & Karin, 2002). Regarding c-Jun/c-Fos dimers, elevation of AP-1 transcriptional activity is partly mediated by an enhanced c-Jun phosphorylation on Ser63 and Ser73 through JNK (Dérijard et al., 1994). This is followed by c-Jun/c-Fos dimerization and binding to AP-1 binding sites in the promoter region of target genes. The *JUN* gene has an AP-1 binding site in its promoter region itself. Therefore, activation of the AP-1 transcription factor leads also to enhanced c-Jun transcription, resulting in a positive feedback loop and elevated levels of the c-Jun protein (Figure 14) (Z. Yang et al., 2009).

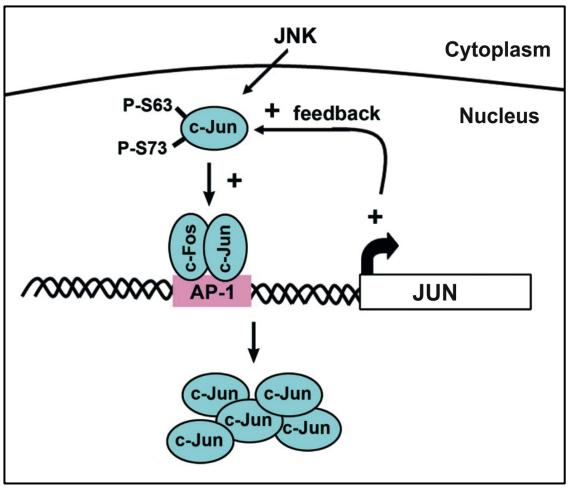

Figure 14: Model depicting the JNK mediated phosphorylation of c-Jun, followed by dimerization with c-Fos and transcriptional activation of *JUN*. JNK, c-Jun N-terminal kinase; AP-1, activator protein 1 (adapted from Z. Yang et al., 2009).

The regulation of MAPK signaling by ROS occurs on different levels. The activation of AP-1 by ROS is mediated mainly by JNK and p38 MAPK cascades (Chang & Karin, 2001). MAPK pathways can be activated by the direct inhibition of MAPK phosphatases by ROS as it was shown for JNK activation (Kamata et al., 2005) and p38 activation (Robinson et al., 1999). Moreover, an upstream MAPKKK that

regulates the JNK and p38 MAPK pathways is the apoptosis signal-regulated kinase 1 (ASK1) (Ichijo, 1997; Tobiume et al., 2001). Reduced thioredoxin was shown to constitutively interact with ASK1, thereby inhibiting its kinase activity (Figure 15). ROS-mediated oxidation of thioredoxin results in dissociation from ASK1, followed by ASK1 activation and subsequent activation of JNK or p38 signaling pathways (Saitoh et al., 1998).

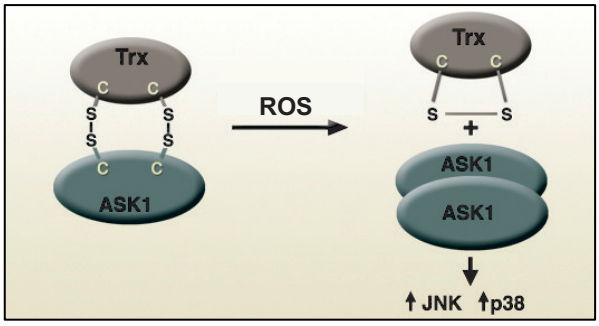

Figure 15: Regulation of ASK1 by ROS. Reduced thioredoxin (Trx) binds and inhibits ASK1. ROS-mediated oxidation of Trx releases ASK1. Activated ASK1 activates downstream effectors such as p38 and JNK. ASK1, apoptosis signal-regulated kinase 1; p38, p38 kinase; JNK, c-Jun N-terminal kinase; ROS, reactive oxygen species (adapted from Finkel, 2011).

1.3.3.6 Nrf2 signaling and its regulation by ROS

A major regulator of antioxidant responses is the nuclear factor (erythroid 2)-like 2 (Nrf2) (J. Zhang et al., 2016). Nrf2 belongs to the cap'n'collar (CNC)-b-zip family of transcription factors. Under non-stimulated conditions, Nrf2 associates with the Kelch-like ECH-associated protein-1 (Keap1) in the cytoplasm (Figure 16). Keap1 interacts with the cullin-3 (Cul3)-containing E3-ubiquitin ligase complex, which results in ubiquitination and proteasomal degradation of Nrf2 (Villeneuve, Lau, & Zhang, 2010). In conditions with elevated ROS levels, cysteine residues in Keap1 become oxidized, leading to the dissociation of Nrf2 from Keap1/Cul3 (D. D. Zhang & Hannink, 2003). Nrf2 becomes stabilized and translocates into the nucleus where it dimerizes with small Maf (musculoaponeurotic fibrosarcoma) proteins (Maf-F, Maf-G and Maf-K). The heterodimer binds to antioxidant response elements (AREs) and activates the transcription of target genes encoding for antioxidant proteins (Kensler, Wakabayashi, & Biswal, 2007). The BTB and CNC homolog 1 (Bach1) is a b-zip transcriptional repressor of the ARE (Ogawa et al., 2001). In the absence of cellular stress, Bach1 heterodimerizes with small Maf proteins and binds to the ARE, thereby repressing gene expression (Figure 16). Elevation of ROS levels cause the translocation of Bach1 to the cytoplasm and subsequent replacement by Nrf2, which

leads to ARE-dependent transcriptional activation (Ishikawa, Numazawa, & Yoshida, 2005; Kaspar & Jaiswal, 2010).

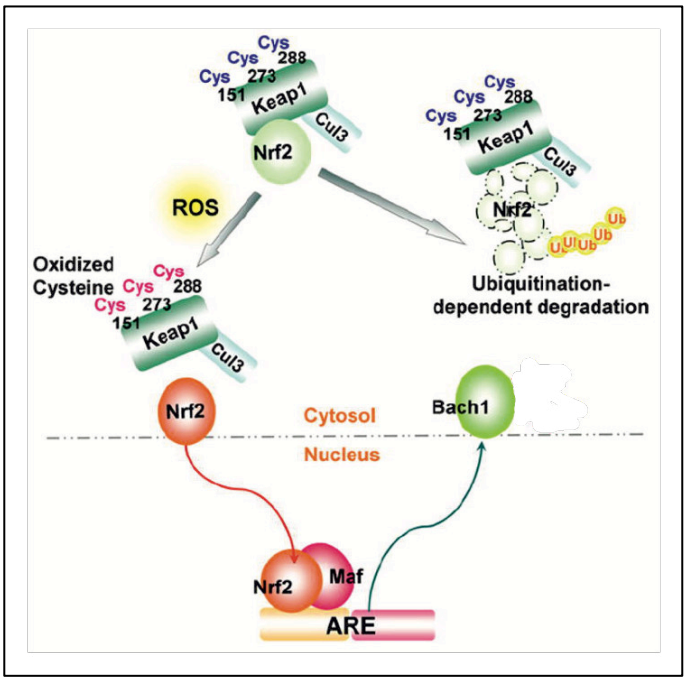

Figure 16: Redox regulation of the Nrf2 signaling pathway. Under non-stimulated conditions, Keap1 associates with Nrf2 in the cytoplasm and targets it for proteasomal degradation following Cul3 mediated polyubiquitination. ROS cause the oxidation of cysteine residues in Keap1, mediating the dissociation of Nrf2. Nrf2 translocates into the nucleus, dimerizes with small Maf proteins and binds to AREs of target genes. Bach1 represses ARE-dependent gene transcription under non-stimulated conditions. ROS lead to translocation of Bach1 from the ARE to the cytoplasm. Nrf2, nuclear factor (erythroid 2)-like 2; Keap1, Kelch-like ECH-associated protein-1; Cul3, cullin-2 E3-ubiquitin ligase; Bach1, BTB and CNC homolog 1; ARE, antioxidant response element (adapted from Ray, Huang, & Tsuji, 2012).

1.3.3.7 NF-κB signaling and its regulation by ROS

The mammalian NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) proteins are of central importance in inflammation and immunity (Q. Li & Verma, 2002). These transcription factors consist of five related family members, which bind as homodimers or heterodimers to specific 10-bp κB sites of target genes (Morgan & Liu, 2011). NF-κB transcription factors are composed of a Rel homology (RHD) domain, which is needed for DNA binding and protein dimerization. RelA (p65), RelB and c-Rel have a C-terminal transcription activation domain (TAD) that is needed to positively regulate gene expression (Hayden & Ghosh, 2008). The two other family members are synthesized as larger p105 (NF-κB1) and p100 (NF-κB2)

precursor proteins. For DNA binding they need to be partially processed by the proteasome to the smaller p50 and p52 products, respectively (Hayden & Ghosh, 2008). p50 and p52 do not activate transcription unless paired as a heterodimer with one of the Rel proteins, as they lack a TAD (Hayden & Ghosh, 2008). NF-κB activity is generally regulated by the inhibitory IκB (Inhibitor of κB) proteins, of which the typical IκBs are IκBα, IκBβ, and IκBε. IκB proteins bind to NF-κB proteins keeping them in the cytosol (Hayden & Ghosh, 2008). Upstream IκB kinases (IKKs) control the activity of the IκBs via phosphorylation (Hayden & Ghosh, 2008). The canonical and the non-canonical pathways are the two main signaling cascades which lead to the activation of NF-κB target genes (Hayden & Ghosh, 2008). The canonical NF-κB pathway is mainly activated by the stimulation of pro-inflammatory receptors (Figure 17) (Q. Li & Verma, 2002). These receptors activate the IKK complex, which consists of the catalytic kinases IKKα and IKKβ, and the regulatory subunit IKKγ (NEMO) (Karin & Ben-Neriah, 2000). The p105 is constitutively processed into p50 (BEINKE & LEY, 2004). p50 is held inactive as a heterodimer with RelA (or c-Rel) by its interaction with the inhibitory IκB proteins. An activated IKK complex phosphorylates IκBα on serines 32 and 36 and targets it for ubiquitination and proteasomal degradation. The p50/RelA heterodimer is released to translocate into the nucleus where it can bind to κB sites and activate gene transcription (Hayden & Ghosh, 2008; Morgan & Liu, 2011; Vallabhapurapu & Karin, 2009).

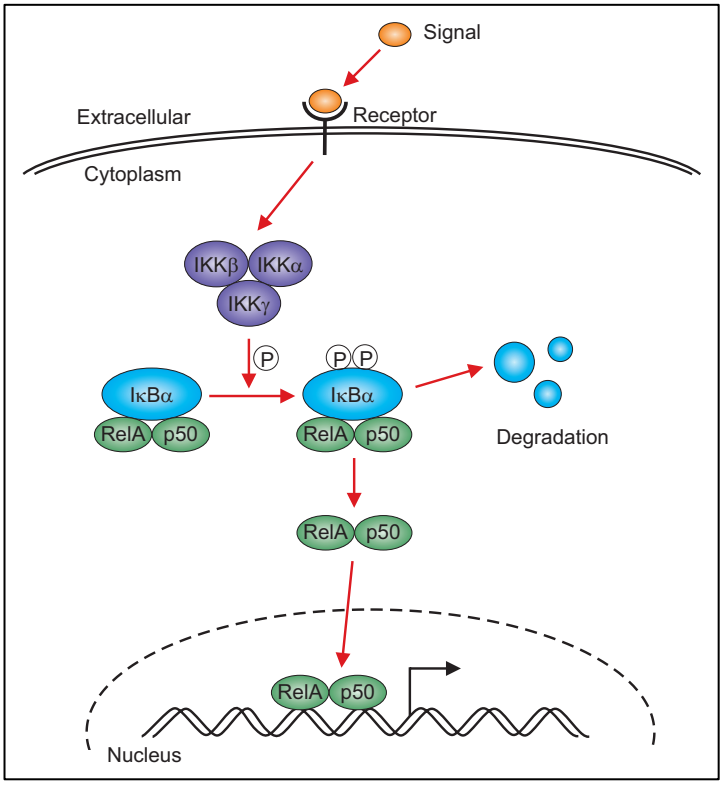

Figure 17: Activation of the canonical NF-κB pathway. Upon stimuli-dependent receptor activation, the IKK complex becomes activated and phosphorylates IκBα. IκBα is targeted for proteasomal degradation, thereby releasing the p50/RelA heterodimer for nuclear translocation and activation of target gene transcription. IκBα, Inhibitor of κB; IKK, IκB kinase.

ROS can interfere at different steps in the NF-κB signaling pathway and can both activate or inhibit NF-κB signaling, depending on the context and also cell type specific (Morgan & Liu, 2011). In cancer cells, NF-κB activation by ROS has been proposed to be mainly regulated via the IKK complexes. For example H_2O_2 has been shown to induce protein kinase D (PKD) phosphorylation, followed by IKKβ phosphorylation and NF-κB activation (Storz, Döppler, & Toker, 2004). Furthermore, various phosphatases are involved in the regulation of NF-κB signaling. For example, phosphorylation and activation of IKKβ has been shown to be reversed by protein phosphatase 2C beta (PP2Cβ), protein phosphatase 1A (PP1A) and protein phosphatase 1B (PP1B) (Prajapati, Verma, Yamamoto, Kwak, & Gaynor, 2004; Sun et al., 2009). Upon oxidation of cysteine residues, these phosphatases may become inactivated, causing a prolonged phosphorylation and activation of IKKβ (Brigelius-Flohé & Flohé, 2011).

Exogenous H_2O_2 and hypoxia/reoxygenation were shown to induce alternative phosphorylation of IκBα on Tyr42, leading to dissociation and activation of NF-κB

through an IKK-independent mechanism (Fan, Li, Ross, & Engelhardt, 2003; Schoonbroodt et al., 2000). In another cell model, H_2O_2 induced IKK-mediated phosphorylation of IκBα at Ser32 and Ser36, followed by NF-κB activation (Gloire et al., 2006), emphasizing that the H_2O_2 dependent NF-κB activation is cell type dependent.

A protein with important roles in tumor progression, which has been shown to be regulated via the NF-κB signaling pathway, is tenascin-C (C. A. Pearson, Pearson, Shibahara, Hofsteenge, & Chiquet-Ehrismann, 1988; Tarassishin, Lim, Weatherly, Angeletti, & Lee, 2014).

1.4 Tenascin-C

Tenascin-C (TNC) belongs to the tenascin family of large extracellular matrix glycoproteins. Vertebrates express four tenascins termed tenascin-C, -R, -X and –W, with each tenascin having a specific expression pattern (Chiquet-Ehrismann & Chiquet, 2003). TNC is highly expressed during embryonic development, but almost absent during postnatal life, only some connective tissues like ligaments, tendons and smooth muscles are positive for TNC (F. S. Jones & Jones, 2000). Further, TNC re-expression occurs under conditions of tissue regeneration such as wound healing, inflammation and in the onset of tumorigenesis (Midwood & Orend, 2009; Tucker & Chiquet-Ehrismann, 2009).

1.4.1 Tenascin-C and cancer

TNC has been linked to cancer since its discovery in the mid-1980s (Bourdon, Wikstrand, Furthmayr, Matthews, & Bigner, 1983; Chiquet, 1984). TNC is overexpressed in many solid tumors (Chiquet-Ehrismann, Mackie, Pearson, & Sakakura, 1986; F. S. Jones & Jones, 2000) and high expression of TNC has been related to poor prognosis (Toshimichi Yoshida, Ishihara, Hirokawa, Kusakabe, & Sakakura, 1995) and local and distant recurrence in breast carcinomas (Jahkola et al., 1998b; Minn et al., 2005). TNC has repeatedly been shown to promote malignant tumor progression and lung metastasis (Midwood, Hussenet, Langlois, & Orend, 2011; Orend, 2005; Oskarsson et al., 2011; Saupe et al., 2013). TNC expression has been linked to ER-negative and in general to invasive tumors of the breast (Dandachi et al., 2001; Ioachim et al., 2002; Shoji et al., 1992). The expression of TNC in early

breast cancer, has been suggested as predictor for an invasive behavior (Goepel, Buchmann, Schultka, & Koelbl, 2000; Jahkola, Toivonen, Nordling, Smitten, & Virtanen, 1998a). Strongest expression of TNC has been found at the invasive front of primary tumors (Orend & Chiquet-Ehrismann, 2006) and of metastases (Oskarsson et al., 2011). TNC is produced by myofibroblasts and angiogenic blood vessels in the cancer associated stroma (Chiquet-Ehrismann et al., 1986; Mackie et al., 1987) and by the cancer cell itself (Toshimichi Yoshida et al., 1997).

1.4.2 Tenascin-C and its role in metastatic progression

TNC has been implicated in various cellular functions which can promote metastasis, such as regulation of adhesion and migration, modulation of immune responses, promotion of angiogenesis and cancer cell-fitness (Lowy & Oskarsson, 2015).
Depending on the occurrence of particular TNC splicing forms and on the overall ECM composition, TNC has been shown to have adhesive or anti-adhesive activities (Midwood & Orend, 2009). For example, TNC inhibits cell adhesion to fibronectin (Chiquet-Ehrismann, Kalla, Pearson, Beck, & Chiquet, 1988), whereby the shortest TNC isoform exhibits the strongest effect (Chiquet-Ehrismann et al., 1991). Furthermore, TNC has been shown to enhance the fitness of disseminated cancer cells and to promote the colonization of secondary organs in mouse models via modulation of Notch and Wnt signaling pathways (Oskarsson et al., 2011). TNC has also been proposed to have a pro-angiogenic role, as shown in experiments with TNC knockout mice where TNC was needed for neovascularization of the embryonic lung and heart (Van Obberghen-Schilling et al., 2011). Furthermore, TNC expression is induced upon vessel damage and is associated with vascular remodeling during dermal tissue repair (Betz, Nerlich, Tübel, Penning, & Eisenmenger, 1993; Fässler, Sasaki, Timpl, Chu, & Werner, 1996; Van Obberghen-Schilling et al., 2011). Additionally, TNC has been associated to the regulation of innate and adaptive immunity. TNC has been shown to activate toll-like receptor (TLR) 4, consequently stimulating the secretion of pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α via NF-κB signaling (Midwood et al., 2009). Thereby, TNC plays an important role in the context of chronic immune diseases (Midwood et al., 2009), but whether TLR4 activation by TNC is involved in tumor progression still needs to be examined. Furthermore, TNC has been shown to impact on the recruitment of macrophages (Talts, Wirl, Dictor, Muller, & Fässler, 1999), and to inhibit T-lymphocyte activation

(Puente Navazo, Valmori, & Ruegg, 2001), indicating that TNC may function as a regulator of inflammatory cells in tumors.

1.4.3 Tenascin-C structure

The human *TNC* gene is a large intron rich gene, located on chromosome 9 at locus 9q32-34/9q33.1 (Gulcher, Alexakos, Le Beau, Lemons, & Stefansson, 1990; Rocchi, Archidiacono, Romeo, Saginati, & Zardi, 1991). *TNC* contains 29 exons of which 9 can be alternatively spliced (Figure 18). The first exon is non-coding and contains the transcription start site. It is separated from exon 2 by a more than 20 kb long intron. Exon 2 contains the ATG start codon for translation initiation (Chiovaro, Chiquet-Ehrismann, & Chiquet, 2015).

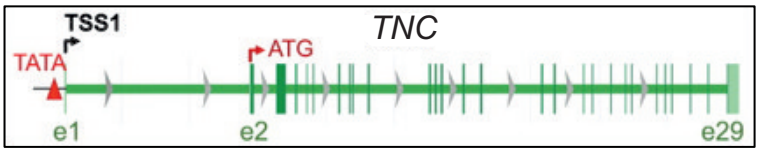

Figure 18: Schematic representation of the *TNC* gene. TSS1, transcription start site; e1, e2, e29, exons 1, 2, and 29; ATG, translation initiation start codon (Chiovaro et al., 2015).

The human tenascin-C protein comprises four domains (Figure 19). The N-terminus contains the assembly domain (AD), which allows disulfide-linked homo-hexamer formation. This is followed by epidermal growth factor-like (EGF-L) repeats and a variable number of fibronectin type III-like (FNIII) repeats. The C-terminus contains a large fibrinogen-like globe (FG) (Chiquet-Ehrismann & Chiquet, 2003). The EGF-L repeats act as a ligand for the EGF-receptor, activating mitogen-activated protein kinase (MAPK) and phospholipase-C (PLC) signaling. The FNIII repeats interact with proteins such as integrins, aggrecan and perlecan, and bind to growth factors like platelet-derived growth factor (PDGF), transforming growth factor-β (TGFβ) and fibroblast growth factor (FGF) (Giblin & Midwood, 2014). The FG domain binds to integrins and activates Toll-like receptor-4 (TLR4) (Giblin & Midwood, 2014; Midwood et al., 2009).

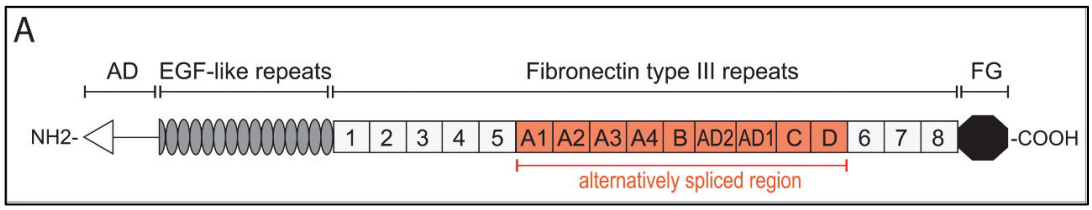

Figure 19: Domain structure of full-length human TNC protein. AD, assembly domain; FG, fibrinogen globe; EGF, epidermal growth factor (Lowy & Oskarsson, 2015).

1.4.4 Transcriptional regulation of tenascin-C

Tenascin-C function is primarily regulated via the control of its expression at the transcriptional level. Many transcription factors have been shown to be involved in transcriptional regulation of *TNC* (Figure 20). TNC transcription may be induced or repressed in response to different stimuli, including inflammatory stimuli (C. A. Pearson et al., 1988; Sarközi et al., 2011; Tarassishin et al., 2014), reactive oxygen species (K. Yamamoto et al., 1999) and mechanical stress (K. Yamamoto et al., 1999).

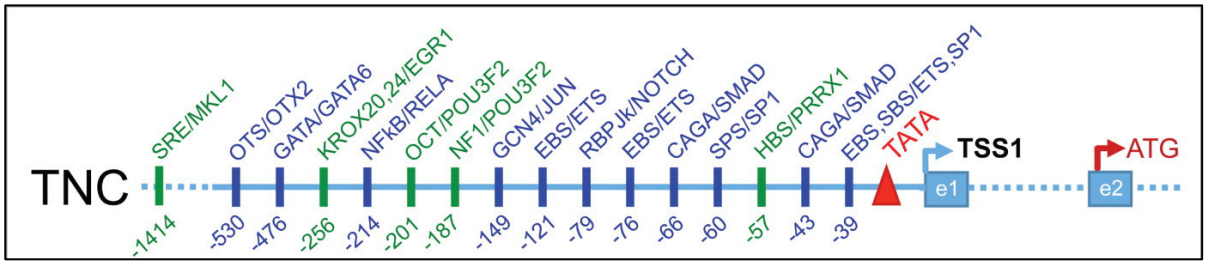

Figure 20: Scheme of the *TNC* gene promoter/ enhancer with experimentally confirmed transcription factor binding sites, reported in human (dark blue) or mouse (green) TNC 3` flanking regions (Chiovaro et al., 2015).

As TNC expression persists in the adult mainly in structures bearing high tensile stress, like ligaments, tendons and the smooth muscle walls of arteries, it is not surprising that TNC expression can be regulated by mechanical stimuli (Crameri et al., 2004; Mackie et al., 1992). External mechanical stimuli signal through integrins, which might cause the activation of ERK1/2, NF-κB or RhoA/ROCK (Sarasa-Renedo & Chiquet, 2005). In rat cardiomyocytes, TNC induction by cyclic mechanical strain was mediated by the release of ROS and the subsequent activation of NF-κB. An NF-κB binding site in the rat *Tnc* promoter was required for transcriptional *Tnc* activation and has been shown to be bound by the p50 subunit of NF-κB upon mechanical stimulation of the cardiomyocytes (K. Yamamoto et al., 1999).

Next to mechanical and oxidative stress, TNC has been shown to be inducible by pro-inflammatory cytokines like IL-1α (Maqbool et al., 2013) and IL-1β (Chevillard, Derjuga, Devost, Zingg, & Blank, 2007; Tarassishin et al., 2014) as well as by anti-inflammatory cytokines like IL-4 (Gratchev, Kzhyshkowska, Utikal, & Goerdt, 2005) and IL-13 (Jinnin et al., 2006), in a cell-type specific manner. In human immune myeloid cells, toll-like receptor (TLR) ligands induced the expression of TNC via activation of NF-κB (Goh, Piccinini, Krausgruber, Udalova, & Midwood, 2010).
Growth factors have also been shown to stimulate TNC gene expression. A direct role of TGF-β in promoting TNC expression has been observed in mammary epithelial cells (HC11) and mouse embryo fibroblasts (Scherberich et al., 2004). In astrocytes, the canonical SMAD-mediated TGF-β signaling pathway has been shown to be involved in TNC regulation (Wiese, Karus, & Faissner, 2012), and in fibroblasts it has been shown that SMADs regulate TNC expression via interaction with cofactors like SP1 and ETS1 in a complex with CBP/p300 (Jinnin et al., 2004).
Transient overexpression of c-Jun induced tenascin-C expression in primary rat embryo fibroblasts and in a rat fibroblast cell line, involving the GCN4/AP1 and NFκB DNA-binding sequences in the *Tnc* promoter (Mettouchi et al., 1997).

1.4.5 Post-transcriptional regulation of tenascin-C

Tenascin-C is regulated post transcriptionally by alternative splicing. The *TNC* gene encodes for numerous different isoforms, generated through alternative splicing of exons within the fibronectin type III (FNIII) repeats (Figure 19) (Lowy & Oskarsson, 2015). TNC isoforms range between 220-320 kDa, while most cancers express the larger isoforms of TNC. Splicing specificity can be context dependent and differs between tumor entities (Lowy & Oskarsson, 2015). In breast cancer, FNIII B and D domains and AD1 are expressed (Adams et al., 2002; Derr, Chiquet-Ehrismann, Gandour-Edwards, Spence, & Tucker, 1997). The regulation of TNC splicing and its relevance in metastasis are generally poorly understood.

1.4.6 Assembly of tenascin-C into a fibrillar matrix

Tenascin-C incorporation into the ECM is dependent on the presence of heparan sulfate proteoglycans (HSPGs) (Chung & Erickson, 1997), fibronectin, α5, αv and β1 integrins (Ramos, Chen, Regezi, Zardi, & Pytela, 1998), and periostin (Kii et al., 2010). The incorporation into a three-dimensional matrix likely changes the

conformation of TNC, exposing or masking specific domains and binding sites. Furthermore, the interaction with other matrix components may affect the availability of binding sites. Hence, the ECM function may differ depending on the conformation of tenascin-C and its interplay with other ECM components (Giblin & Midwood, 2014).

1.4.7 Proteolytic processing of tenascin-C

Tenascin-C function is additionally regulated via proteolytic cleavage. TNC can be degraded in situations where a transient expression is required, such as in healing wounds, or proteolysis can release smaller fragments with different functions (Giblin & Midwood, 2014). TNC is susceptible to cleavage by matrix metalloproteinases (MMPs) and serine proteases (Imai, Kusakabe, Sakakura, Nakanishi, & Okada, 1994; Siri et al., 1995), whereby different splicing forms show distinct sensitivity to specific proteases. For example, MMP2 and MMP3 cleave the large TNC isoform (Siri et al., 1995) exclusively at the FNIIIA3 domain (S. C. Bell, Pringle, Taylor, & Malak, 1999). MMP2 mediated cleavage generates a “cryptic binding site” (G. E. Davis, Bayless, Davis, & Meininger, 2000) within FNIII A2, exerting a pro-adhesive effect via $\alpha 5\beta 1$ integrin activation (Saito et al., 2007; Tanaka et al., 2014).

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2. Aims of the thesis

The aims of the thesis were:

1. To examine the relevance of chronic intermittent hypoxia for the modulation of gene expression in breast cancer cell lines.
2. To elucidate the molecular mechanisms, which are involved in the modulation of gene expression upon chronic intermittent hypoxia.
3. To analyze the effect of chronic intermittent hypoxia on breast cancer cell malignant properties.

3. Manuscript: Intermittent hypoxia confers pro-metastatic gene expression selectively through NF-κB in inflammatory breast cancer cells

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Running title: IH regulates pro-metastatic gene expression in IBC

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ABSTRACT

Inflammatory breast cancer (IBC) is the most aggressive form of breast cancer. Treatment options are limited and the mechanisms underlying its aggressiveness are poorly understood. Intermittent hypoxia (IH) causes oxidative stress and is emerging as important regulator of tumor metastasis. Vessels in IBC tumors were shown to be immature, which is a primary cause of IH. We therefore investigated the relevance of IH for the modulation of gene expression in IBC cells in order to assess IH as potential regulator of IBC aggressiveness. Gene array analysis of IBC cells following chronic IH (45-60 days) demonstrated increased expression of pro-metastatic genes of the extracellular matrix, such as tenascin-C (TNC; an essential factor of the metastatic niche), and of inflammatory processes. Investigating the oxidative stress-dependent regulation of TNC, we found a gradual sensitivity on mRNA and protein levels. Oxidative stress activated nuclear factor erythroid 2-related factor 2 (Nrf2), c-Jun N-terminal kinase (JNK), c-Jun and nuclear factor κB (NF-κB), but TNC upregulation was only dependent on NF-κB activation. Pharmacological inhibition of inhibitor of NF-κB α (IκBα) phosphorylation and overexpression of IκBα prevented TNC induction, whereas the proinflammatory cytokine interleukin-1β (IL-1β) increased TNC expression. Analysis of the gene array results showed NF-κB binding sites for 64% of all upregulated genes, linking NF-κB and IH-dependent regulation of pro-metastatic gene expression in IBC cells. Our results provide a first link between intermittent hypoxia and pro-metastatic gene expression in IBC cells, revealing a putative novel mechanism for the high metastatic potential of IBC.

HIGHLIGHTS

- Intermittent hypoxia (IH) increases pro-metastatic gene expression in IBC cells
- ROS activates NF-κB, Nrf2 and c-Jun in IBC cells
- IH-dependent gene regulation depends on NF-κB but no other redox signaling pathways
- IH is proposed as an important regulator of IBC aggressiveness

INTRODUCTION

Inflammatory breast cancer (IBC) is the most aggressive and most lethal form of primary breast cancer. While IBC is estimated to account for up to 5% of all breast cancer cases, it leads to approx. 8-10% of all breast cancer-related deaths [1-3]. IBC presents with a diffuse tumor formation and is highly metastatic with lymph node metastases being found in almost all and distant metastases in 30% of patients, respectively, at the time of diagnosis [2]. Treatment of IBC patients has proven to be challenging due to its rapid progression and aggressive nature [2, 4]. The mechanisms underlying the aggressiveness of IBC are still poorly understood, impeding the development of targeted therapy [2, 5]. In addition, the investigation of molecular signaling pathways leading to the development of the aggressive phenotype of IBC is difficult, because of a limited availability of tumor material and with only two primary tumor-derived cell lines existing [5, 6].

Tumors frequently outgrow their blood oxygen supply, leading to insufficient oxygen levels (hypoxia) and subsequent induction of angiogenesis [7]. The induced tumor vessels are often not properly developed, leading to fluctuations of blood and hence oxygen supply [8]. Oxygen levels in solid tumors are therefore dynamic, with repeated cycles of hypoxia and reoxygenation, called chronic intermittent hypoxia (CIH) or cycling hypoxia [8]. CIH, although not directly assessed yet in IBC, likely also occurs in IBC tumors, because analysis of IBC tumor tissue showed hypoxia-dependent gene expression (carbonic anhydrase IX (CA9) induction) in 50% of the tumors and overall a higher microvessel density than in other breast cancer forms [4, 9, 10]. Moreover, approx. 90% of IBC tumor blood vessels are not fully developed [11]. Of note, CIH leads to enhanced tumor microvessel density, which could present a link to the increased microvessel density in IBC tumors [12]. In addition, CIH has been shown to be a stronger inducer of cancer metastases than continuous hypoxia [13, 14]. However, the role of CIH in IBC progression and aggressiveness is unclear.

CIH is a major source of oxidative stress due to reactive oxygen species (ROS) generation [8, 15, 16]. Oxidative stress can affect gene expression through regulation of DNA methylation and transcription factor activity [15, 17, 18]. While the mechanism of oxidative stress-dependent regulation of DNA methylation is less clear, several transcription factors have been reported to be activated by oxidative stress, including nuclear factor erythroid 2-related factor 2 (Nrf2), hypoxia-inducible factor (HIF), activator protein-1 (AP-1) and nuclear factor κB (NF-κB) [15, 18, 19].

Nrf2 is the main regulator of the cellular antioxidant response [20]. HIF is the master regulator of the transcriptional response to hypoxia but its activation by oxidative stress is still highly debated [8, 21-24]. AP-1 has been linked to the regulation of a wide range of cellular processes, including proliferation, migration and inflammation and has been described to play an important role in tumor cell proliferation in the context of oxidative stress [25, 26]. NF-κB is the master regulator of the cellular transcriptional response to inflammation [27, 28]. In cancer cells, NF-κB activity can increase the expression of genes that promote proliferation, invasiveness, metastasis and apoptosis [29, 30].

The extracellular matrix (ECM) plays an important role in the development and progression of breast cancer [31]. For example, the matrix metalloproteinases 2 and 9 (MMP2, MMP9) and a disintegrin and metalloproteinase 19 (ADAM19) are remodeling enzymes of the ECM and play a major part in the development of tumor metastasis and invasion [31, 32]. Tenascin-C (TNC), a large hexameric ECM glycoprotein, is highly expressed during development, almost absent during postnatal live, but re-expressed in case of injury and in the onset of tumorigenesis [33]. Increased expression of TNC has been related to poor prognosis, local and distant recurrence in breast carcinomas and metastasis formation [31, 34]. Of note, TNC has been shown to be an essential factor of the metastatic niche [31].

In the current study, we investigated the impact of intermittent hypoxia and the resulting oxidative stress on transcriptional regulation in IBC cells. We provide evidence that CIH and oxidative stress are important regulators of pro-metastatic gene expression in IBC cells. Investigation of the underlying mechanism of upregulated TNC gene expression showed that it is dependent on NF-κB activation but not on DNA methylation, Nrf2, AP-1 and HIF. Furthermore, 64% of all CIH-upregulated pro-metastatic genes can be regulated by NF-κB. These data provide a mechanistic link between intermittent hypoxia and pro-metastatic gene expression in IBC cells, contributing insights into the regulation of IBC aggressiveness.

MATERIAL AND METHODS

Reagents

Tert-butylhydroquinone (30 μM), dimethyl fumarate (40 μM), BAY11-7082 (12.5 μM, 25 μM) and H_2DCFDA (10 μM; Invitrogen) were dissolved in ethanol. L-Sulforaphane (10 μM) and SP600125 (10 μM, 20 μM) were dissolved in DMSO and N-acetyl-L-cysteine (3 mM, 20 mM) was dissolved in H_2O. IL-1β was dissolved in 0.1% BSA in PBS.

Cell culture and treatments

The human triple-negative breast cancer cell line SUM149PT was kindly provided by Rachael Natrajan (London, UK) and was used for all subsequent cell culture experiments. SUM149PT cells were cultured in equal parts of high-glucose DMEM (Sigma-Aldrich) and Ham's F-12 Nutrient Mixture (Gibco, Thermo Fischer Scientific, Waltham, MA, USA), supplemented with 5% heat-inactivated fetal bovine serum (FBS), 1 μg/ml hydrocortisone (Sigma-Aldrich), 5 μg/ml human insulin (Sigma-Aldrich), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich). Reagents were diluted to the required concentration in DMEM/F-12 medium without FBS and supplements. SUM149PT cells were seeded at 70-80% confluency. BAY11-7082, SP600125 and N-acetyl-L-cysteine were added to the cells 1 h before addition of tBHQ. Hypoxic experiments were carried out in a humidified atmosphere containing 0.2% O_2 and 5% CO_2 in a gas-controlled glove box (Invivo2 400, Baker Ruskinn, Bridgend, UK) as previously described [35].

mRNA analysis and data deposition

The RNeasy mini kit (Qiagen, Hilden, Germany) was used for total RNA extraction for the Affymetrix gene array analysis according to manufacturer's protocol. Affymetrix Human Gene 2.1 ST strip arrays were used to analyze transcript levels of one biological replicate of untreated cells and of cells cultured for 45 days in CIH. The data were deposited in Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through the ID GSE81416. For validation of the Affymetrix gene array results three biological replicates of untreated cells and cells that were cultured for 45 days in normoxia or CIH conditions were analyzed by RT-qPCR as previously described [36]. The relative mRNA expression was calculated using the $\Delta\Delta C_t$ formula. U6 snRNA served as control gene and values

were normalized to the average values of normoxic controls. For all further experiments, total cellular RNA was extracted as previously described [37]. Primer sequences were listed in Supplementary Table S3. mRNA copy numbers were calculated by comparison with serial dilutions of a calibrated standard, calculated relative to U6 snRNA levels and normalized to starting time point if not otherwise indicated.

Bioinformatic analysis of Affymetrix gene expression array

For the analysis of the Affymetrix gene array results, protein coding genes, as listed in the National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) database, with ≥ 4-fold increased transcript levels were used for bioinformatic analyses with MetaCore (https://portal.genego.com/) from Thomson Reuters. Enrichment analysis according to standard settings of the software was performed to rank the most relevant cellular processes. p values were calculated as –log10. To analyze literature-based transcriptional regulations the “build network” option with the algorithm for “transcription regulation” was applied. In order to link single genes to the matrisome, analysis was performed via the matrisome project for each upregulated gene (http://matrisomeproject.mit.edu/proteins/) [38]. For detection of conserved transcription factor binding sites (TFBS), the web-based software oPOSSUM version 3 (http://opossum.cisreg.ca/oPOSSUM3/) was used [39-41]. The matrices of TFBS profiles included in oPOSSUM-3 were obtained from the 2010 release of the JASPAR database (http://jaspar.genereg.net). For in silico analysis of expression data the R2 database (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi) was searched across datasets and 2D gene overview options were used for correlations between TNC and p105 in Affymetrix HG-U133plus2.0-based expression profiles normalized using MAS5.0. The following GEO IDs were employed for our analyses: GSE76124, GSE21653, GSE3494, GSE25066. For detection of TFBS’ in the *TNC* promoter, the UCSC genome browser (http://genome.ucsc.edu/) was searched with the genome assembly released in February 2009 [42]. Further, the Swissregulon (http://swissregulon.unibas.ch/fcgi/sr) and the JASPAR databases [43] were searched.

Protein detection and quantification

Proteins were extracted from cells using 0.4 M NaCl, 1% Nonidet P-40, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium-orthovanadate and protease inhibitor cocktail (P2714, Sigma-Aldrich). Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and detected with antibodies against HIF-1α (BD Transduction Laboratories, Allschwil, Switzerland), β-actin (Sigma-Aldrich), SMC1 (Abcam, Cambridge, UK), tenascin-C (H-300, Santa Cruz Biotechnology, Dallas, TX, USA), Nrf2 (H-300, Santa Cruz Biotechnology), α-tubulin (Sigma-Aldrich), c-Jun (Cell Signaling Technologies, Danvers, MA, USA), phospho-c-Jun(Ser63) (Cell Signaling Technologies), phospho-c-Jun(Ser73) (Cell Signaling Technologies), IκBα (Cell Signaling Technologies), phospho-IκBα (Cell Signaling Technologies), and Nrf3 [44]. Quantification of immunoblotting signals were performed by ImageJ 1.48v (http://imagej.nih.gov/ij) or as previously described [35]. Values were normalized to β-actin loading control and to the average values of normoxic controls if not otherwise indicated.

siRNAs and plasmids

For siRNA experiments, cells were seeded to reach 60% confluency on the day of transfection. Nrf2 siRNA (ON-TARGET plus Human NFE2L2 siRNA, SMART pool), Nrf3 siRNA (ON-TARGET plus Human NFE2L3 siRNA, SMART pool), c-Jun siRNA (ON-TARGET plus Human JUN siRNA, SMART pool) and non-target siRNA (ON-TARGET plus Non-targeting Pool) were purchased from Dharmacon (GE Healthcare, Lafayette, CO, USA) and transfected using Lipofectamine 2000 (Invitrogen) and Opti-MEM (GIBCO) according to manufacturers' protocols. For plasmid transfections, cells were seeded to reach 70% confluency on the day of transfection. NC16 pcDNA3.1 FLAG Nrf2 was a gift from Randall Moon (Addgene plasmid # 36971) [45], pCMV4-3 HA/IκBα was a gift from Warner Greene (Addgene plasmid # 21985) [46] and pcDNA3.1-hNFE2L3 has previously been published [44]. Following 24 h of transfection, treatments were performed.

H_2DCFDA assay

SUM149PT cells were seeded in 96-well plates at 80% confluency and incubated with 10 μM 2'7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Invitrogen) for 30

min in the dark. Subsequently, specific treatments were performed and fluorescence was measured using a 96-well fluorescence photometer (Infinite 200Pro, Tecan, Männedorf, Switzerland). For combined treatments with tert-butylhydroquinone and N-acetyl-L-cysteine, cells were pretreated for 1 h with N-acetyl-L-cysteine. Results were calculated as increase in fluorescence per well ((Ftx – Ft0) / Ft0*100), where Ftx = fluorescence at a certain time point and Ft0 = fluorescence at 0 min [47]. For hypoxia-reoxygenation experiments, 96-well plates were incubated for 24 h in 0.2% oxygen. Cells were incubated with H_2DCFDA inside the hypoxia workstation and fluorescence measurements were performed immediately thereafter.

DNA methylation analyses

Quantification of CpG density in the genomic sequence of *TNC* was carried out using MethPrimer Software (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) [48]. Genomic DNA was treated with bisulfite prior to methylation-specific PCR (MSP) or bisulfite sequencing PCR (BSP) using the EZ-DNA methylation-Gold kit (Zymo Research Corp, Irvine, CA, USA) as per manufacturer's description. MSP was performed on 5 ng bisulfite-treated DNA using the outlined primers (Supplementary Table S3), targeting CpG island in the *TNC* promoter region [49]. BSP was performed using M13-tailed primers (primers designed: MethPrimer software), targeting the CpG island in the promoter region or the first untranslated exon, respectively, of the *TNC* gene.

Statistical analyses

If not otherwise indicated, results are presented as mean values ± standard error of the mean (SEM) of at least three independent experiments. Statistical analyses were performed using Student's t-test and one-way ANOVA or two-way ANOVA where appropriate. P-values <0.05 were considered statistically significant.

RESULTS

Chronic intermittent hypoxia increases pro-metastatic gene expression in inflammatory breast cancer cells.

In order to investigate the impact of CIH on IBC, the primary tumor-derived IBC cell line SUM149PT was cultured for 45 days in CIH conditions (15 cycles of hypoxia/reoxygenation; Fig. 1A) and gene expression was assessed by whole genome microarray hybridization. Using a four-fold difference relative to control as threshold for relevant gene regulation, 37 genes were identified to be upregulated and 51 genes to be downregulated in response to CIH. Analysis of associations of the upregulated genes with cellular processes via MetaCore (https://portal.genego.com/) showed that these genes are primarily involved in the regulation of the ECM (Fig. 1B). 13 out of the 37 upregulated genes were linked to the “matrisome”, which is composed of ECM and ECM-associated proteins (Fig. 1B, Supplementary Table S1) [38]. A second relevant process associated with CIH-upregulated gene expression was inflammation (Fig. 1B).

The CIH experiment was repeated to validate the expression levels of 10 out of the 37 upregulated genes. TNC, MMP2, MMP9 and ADAM19 were chosen because these proteins are important components and remodeling enzymes of the ECM, and since they have previously been shown to play a major role in tumor invasion and metastasis [31, 32]. Triggering receptor expressed on myeloid cells (TREM1), interleukin-1α (IL1A) and cyclooxygenase-2 (COX-2; gene name: *PTGS2*, prostaglandin G/H synthase 2) have been linked before to inflammatory processes and COX-2 has been associated with a more aggressive breast cancer phenotype [2]. nuclear factor erythroid 2-related factor 3 (Nrf3, gene name: *NFE2L3*) is a homolog of the transcription factor Nrf2 and might be part of the oxidative stress response [50]. Src homology 2 domain containing family member 4 (SHC4) plays a critical role in migration of metastatic melanomas and potentially in the response to oxidative stress-induced DNA damage; and a SNP in X-ray repair complementing defective repair in Chinese hamster cells 2 (XRCC2), which is involved in homologous recombination DNA repair, was linked to spontaneous breast cancer development [51-53]. We analyzed the expression pattern of these genes in response to hypoxia, reoxygenation (both part of cycle 1 of the CIH experiment; Fig. 1A) and CIH (15-20 cycles; Fig. 1A). All genes except MMP9 demonstrated a significant upregulation following CIH (Fig. 2A). But also MMP9 showed an

upregulation in response to CIH in all three independent experiments, only with a higher degree of variability. To analyze the expression patterns further, we directly compared the observed induction factors of the different treatments to each other and identified three differentially regulated groups (Fig. 2B). Group I showed only upregulated gene expression in response to CIH, while group II genes were already increased in response to a single reoxygenation stimulus (Fig. 2B). Group III was upregulated by all three treatments (Fig. 2B). These findings indicate that some of these genes are only sensitive to a higher oxidative stress such as CIH (group I), while others are already upregulated by a single reoxygenation event (group II). In group III, we most likely observed overlapping regulation between hypoxia and the oxidative stress response. Of note, no increase in prototypical HIF target genes (e.g. CA9 or glycolytic enzymes) was observed in the gene array following CIH, indicating that HIF-dependent gene expression was not present in our CIH conditions at the time of analysis. In order to determine if CIH also led to increased protein levels, we determined TNC protein expression following the three different treatments. TNC protein expression followed the same pattern as its mRNA, with no upregulation in response to hypoxia or a single reoxygenation event but with a significant increase following 15 and 20 cycles of CIH (Fig. 2C, D).

Overall, these results demonstrate that CIH increases pro-metastatic gene expression in IBC cells. In addition, we identified three distinct groups of regulated genes, indicating a differential sensitivity to oxidative stress and hypoxia among the investigated genes. The observed changes on mRNA levels translated into protein expression, indicating functional relevance.

Tenascin-C expression demonstrates a gradual sensitivity to increasing oxidative stress levels

We next investigated the underlying mechanism of the CIH-dependent increase in pro-metastatic gene expression. We focused on TNC expression because its mRNA and protein levels were specifically upregulated in response to CIH and not by hypoxia, demonstrating that its regulation depends on repeated reoxygenation events (Fig. 2). Furthermore, TNC has been linked to breast cancer development, it plays a major role in tumor progression and metastasis, and it has been shown to be an essential factor of the metastatic niche [31]. Because CIH is a well-established oxidative stress inducer and likely leads to a more severe oxidative stress than a

single reoxygenation event [8, 15, 54], we aimed to investigate the effect of increasing oxidative stress conditions on TNC-dependent gene expression. First, we compared the potential of different stimuli, a single hypoxia/reoxygenation phase, hydrogen peroxide (H_2O_2; Sigma-Aldrich, St Louis, MO, USA) and tert-butylhydroquinone (tBHQ; Sigma-Aldrich), to induce oxidative stress in SUM149PT cells using the H_2DCFDA assay (Invitrogen, Thermo Fischer Scientific, Waltham, MA, USA) (Fig. 3A-C). Hypoxia/reoxygenation led to a short but significant increase in DCF fluorescence at early time points (0.5 h) (Fig. 3A), while 100 μM H_2O_2 led to a prolonged, plateauing increase in fluorescence over 4 h (Fig. 3B). 30 μM tBHQ induced a steady increase in fluorescence over 4 h and the observed signal was up to 4-fold higher than with single hypoxia/reoxygenation or H_2O_2 (Fig. 3A-C). This established the following order of ROS induction by these treatments: tBHQ (30 μM) > H_2O_2 (100 μM) > single reoxygenation.

Next, we investigated the impact of the different ROS sources on TNC mRNA and protein expression. Hypoxia/reoxygenation led to a significant but short and transient increase in TNC mRNA levels at 2 and 4 h (Fig. 3D), while H_2O_2 significantly induced TNC mRNA at 4-8 h (Fig. 3E) and tBHQ at 9 h and 24 h (Fig. 3F), with 5 to 9-fold higher TNC mRNA levels following tBHQ treatment compared to the other treatments (Fig. 3D-F). On the protein level, hypoxia/reoxygenation neither induced TNC (Fig. 3G, J) nor activated the positive control for oxidative stress, the redox-sensitive transcription factor Nrf2 (data not shown). CA9 mRNA levels and HIF-1α protein levels were used as positive controls for hypoxia (Fig. 3G, Supplementary Fig. S1A). In turn, H_2O_2 increased TNC protein levels significantly after 6 h (Fig. 3H, K). Nrf2 protein was stabilized and its target gene heme oxygenase 1 (HO-1) was elevated in response to H_2O_2 treatment (Fig. 3H, Supplementary Fig. S1B). tBHQ treatment led to a strongly increased protein expression at 9 h and 24 h (Fig. 3I, L). Nrf2 protein levels and HO-1 mRNA were also elevated by tBHQ (Fig. 3I, Supplementary Fig. S1C). Of note, the observed differences of activated Nrf2 protein levels between the treatments are in agreement with the differences of ROS measured by the H_2DCFDA assay (tBHQ > H_2O_2 > single reoxygenation), further supporting a differential ROS induction between the treatments (Fig. 3A-C, G-I).

Overall, TNC expression shows a gradual sensitivity to ROS levels in inflammatory breast cancer cells, indicating that CIH increases TNC expression through increasing oxidative stress.

Antioxidant treatment prevents oxidative stress-mediated TNC induction

We next sought to determine if the observed TNC induction was directly dependent on ROS. SUM149PT cells were pre-treated with the antioxidant N-acetyl-L-cysteine (NAC; Sigma-Aldrich) for 1 h followed by the addition of tBHQ. NAC prevented tBHQ-mediated ROS production (Fig. 4A) as well as tBHQ-mediated upregulation of TNC protein levels (Fig. 4B and C). These results demonstrate that regulation of TNC expression is directly linked to oxidative stress and can be prevented by antioxidant treatment.

Chronic intermittent hypoxia-dependent TNC induction is not regulated by changes in DNA methylation

DNA methylation is frequently involved in chronic changes of gene expression and can be regulated by oxidative stress [17]. Therefore, we investigated a potential impact of CIH on DNA methylation as molecular mechanism for the regulation of TNC expression. First, the regions upstream and downstream of the transcriptional start site of the *TNC* gene were analyzed with the MethPrimer software (http://www.urogene.org/methprimer/) [48]. This identified a CpG-rich region in the *TNC* promoter and in the first untranslated exon of *TNC* (Fig. 5A). Analysis by methylation-specific PCR (MSP) showed no difference in methylation of the *TNC* promoter following CIH (Fig. 5B). Analysis by bisulfite sequencing PCR (BSP) detected also no change in methylation after CIH, neither in the *TNC* promoter nor in the first exon of *TNC* (data not shown). Hence, the observed elevated TNC expression upon CIH is not caused by changes in DNA methylation.

Oxidative stress-dependent TNC induction is independent of Nrf2 and Nrf3

A main transcription factor activated by oxidative stress is Nrf2 [20] and Nrf2 protein was stabilized and Nrf2-dependent target genes were increased in SUM149PT cells following H_2O_2 and tBHQ treatment (Fig. 3H, I, Supplementary Fig. S1B, C). In addition, in response to CIH we observed elevated Nrf3 mRNA levels (Fig. 2A). Nrf3 belongs to the same transcription factor family as Nrf2 (Cap'n'Collar family) and has

also been linked to the cellular antioxidant response [50]. Furthermore, the *TNC* promoter can be regulated by BACH1, a transcriptional suppressor binding antioxidant response elements (ARE) (Fig. 6A) [55]. Following oxidative stress, BACH1 does not suppress ARE-dependent transcription anymore [55], so that for example Nrf2 or Nrf3 can bind to regulate gene expression (Fig. 6A). Therefore, we transiently knocked down Nrf2 and/or Nrf3 in SUM149PT cells, followed by tBHQ treatment. No significant difference in tBHQ-dependent TNC induction was detected with single or combined Nrf2 and Nrf3 knockdown (Fig. 6B-D), while HO-1 induction, a known direct Nrf2 target gene, was significantly affected (Fig. 6B-D) [20]. Single and combined knockdown or overexpression of Nrf2 and Nrf3 showed also no major effect on basal TNC gene expression (Supplementary Fig. S2). In addition, the effect of two chemical Nrf2 activators on TNC expression was tested, L-sulforaphane (SFN; Sigma-Aldrich) and dimethyl fumarate (DMF; Sigma-Aldrich). SFN and DMF mediate stabilization and nuclear translocation of Nrf2 by modulating the inhibitory protein of Nrf2, kelch-like ECH-associated protein 1 (Keap1) [56, 57]. SUM149PT cells were treated with 10 μM SFN and samples were collected for up to 24 h. No change in TNC mRNA expression was detected, while the induction of HO-1 6 h after treatment demonstrated efficient Nrf2 activation (Fig. 6E). Treatment with 40 μM DMF caused only a slight elevation in TNC mRNA levels after 24 h, but showed a significant HO-1 induction from 3 h to 9 h (Fig. 6F). Increased TNC mRNA levels through DMF stimulation correlated with increased ROS levels while SFN caused no significant ROS production and no elevated TNC mRNA levels (Supplementary Fig. S3). Of note, the increase in ROS following DMF treatment was much lower than the ROS levels measured after H_2O_2 treatment and negligible compared to the ROS production caused by tBHQ (Fig. 3B, C, Supplementary Fig. S3).

Overall, these results demonstrate that oxidative stress-dependent TNC induction is independent of Nrf2 and Nrf3.

Oxidative stress-dependent TNC induction is independent of JNK and c-Jun activity.

Another transcription factor activated by oxidative stress and IH is AP-1, which is regulated by mitogen-activated protein kinase (MAPK) signaling [15, 18, 25]. AP-1 is a dimeric protein that can be composed of several different subunits of which the major protein subfamilies are Jun (c-Jun, JunB, JunD) and Fos (c-Fos, FosB, Fra-1,

Fra-2) [25]. The *TNC* promoter contains ChIP-based binding sites for AP-1 subunits such as c-Jun (Fig. 6A). tBHQ increased over a time course of 24 h c-Jun phosphorylation on serine 63 and 73, which enhances c-Jun transcriptional activity (Fig. 7A) [25]. In addition, total c-Jun protein levels were increased (Fig. 7A). c-Jun has been reported to be phosphorylated on serine 63 and 73 by the MAPK c-Jun N-terminal kinase (JNK) [25]. Therefore, we next investigated if tBHQ-dependent c-Jun phosphorylation could be prevented by the JNK inhibitor SP600125 (Selleck, Houston, TX, USA). One hour pre-treatment with SP600125 blocked tBHQ-dependent c-Jun phosphorylation (Fig. 7B), indicating that tBHQ activates the MAPK signaling cascade at the level of or upstream of JNK. However, the JNK inhibitor did not prevent tBHQ-mediated TNC induction (Fig. 7C, D). Furthermore, siRNA-mediated knockdown of c-Jun did also not significantly affect tBHQ-dependent TNC induction (Fig. 7E, F). These results show that oxidative stress activates MAPK signaling and c-Jun in IBC cells but the oxidative stress-dependent increase of TNC is independent of this mechanism.

Oxidative stress-dependent TNC induction is mediated by NF-κB.

A further transcription factor activated by oxidative stress is NF-κB, which also has a putative binding site in the *TNC* promoter (Fig. 6A). Hence, we next tested if tBHQ can activate NF-κB in IBC cells and treated SUM149PT cells with tBHQ over a 24 h time course. This led to a time-dependent decrease of inhibitor of NF-κB α (IκBα) protein levels (Fig. 8A), demonstrating activation of the NF-κB signaling pathway. Pre-treatment with BAY11-7082 (Selleck), which prevents phosphorylation and subsequent degradation of IκBα [58], blocked the tBHQ-dependent decrease of IκBα (Fig. 8B), indicating that tBHQ-mediated oxidative stress activates the NF-κB signaling pathway at the level of or upstream of IκBα phosphorylation. Inhibitor treatment 1 h prior to the tBHQ stimulus abrogated the increase in TNC expression (Fig. 8C, D), indicating that NF-κB regulates oxidative stress-mediated TNC induction. Overexpression of IκBα also prevented oxidative stress-dependent induction of TNC, confirming its regulation by the NF-κB signaling pathway (Fig. 8E).

Treatment with the major pro-inflammatory cytokine IL-1β (R&D systems, Minneapolis, MN, USA), one of the best-characterized NF-κB activating stimuli [58, 59], showed a decrease of IκBα levels at 30 min and a strong increase in TNC

protein levels from 3-24 h (Fig. 8F). Analysis of breast cancer transcriptomic datasets revealed a significant, positive correlation between TNC and nuclear factor κB p105 subunit (p105) levels (Supplementary Fig. S4). Together with the previous results, this demonstrates that oxidative stress-induced TNC expression is regulated by NF-κB. Interestingly, when we investigated our CIH gene array results for transcription factor binding sites and known regulation by transcription factors using oPOSSUM and MetaCore (http://opossum.cisreg.ca/oPOSSUM3/ and https://portal.genego.com/), 64% of all upregulated genes showed a possible regulation by NF-κB (Fig. 8G, Supplementary Table S2). Analysis for binding sites for AP-1 and Nrf2 revealed a putative regulation of 33% by Nrf2 and of 83% by AP-1 with 30.6% of these genes showing overlapping regulation by Nrf2, AP-1 and NF-κB (Supplementary Fig. S5).

Overall, our results demonstrate that oxidative stress-dependent TNC increase is specifically regulated by NF-κB and that this transcription factor might also play an important role in the upregulation of other pro-metastatic genes in IBC cells in response to CIH.

DISCUSSION

Inflammatory breast cancer is the primary breast cancer form with the highest aggressiveness [2, 3, 60]. The underlying mechanisms are still unclear but are urgently needed to be deciphered in order to develop novel treatment options [5]. CIH is a common feature of tumors, but it is only beginning to be appreciated as an important regulator of cancer progression and metastases. It occurs when cancer cells outgrow the area of sufficient oxygen delivery and subsequent hypoxia-induced tumor angiogenesis leads to vessels with abnormal structures [8]. Such changes have also been demonstrated for IBC tumors [7, 11]. CIH is a well-established source for oxidative stress, which can severely impact on gene expression, leading to increased cancer aggressiveness [16]. However, the relevance of CIH for IBC progression and metastases formation is unclear. In this study, we provide insights into the regulation of pro-metastatic gene expression through IH and oxidative stress in IBC cells, which appears to be mediated mainly by NF-κB (Fig. 9).

In our investigations, we identified that CIH leads to increased expression of ECM proteins in IBC cells, such as TNC, MMP2, MMP9 and ADAM19, and of proteins involved in inflammatory processes, such as COX-2. TNC, MMP2, MMP9 and COX-2 have previously been shown to highly promote breast cancer metastases, and to be significantly stronger expressed in IBC tumors in comparison to non-IBC breast cancer tumors [2, 31, 60, 61]. Tumor inflammation has also been associated with cancer progression and metastasis formation and COX-2 was identified as a major component of the IBC molecular signature and suggested for targeted therapy for IBC [2, 62]. Overall, these data demonstrate that CIH leads to the upregulation of pro-metastatic gene expression in IBC cells, which likely contributes to the aggressive phenotype of IBC.

The transcriptional regulation of the TNC gene is complex and several different stimuli and transcription factors are involved [63]. TNC has previously been shown to be regulated by AP-1, NF-κB and Nrf2 [63-65], and although we observed a ROS-dependent activation of Nrf2, JNK and c-Jun in IBC cells, these factors did not regulate TNC. In our gene array, 33% of CIH-upregulated genes were identified to carry a putative Nrf2 binding sequence, but we did not observe any prototypical Nrf2 target gene, such as HO-1 or other genes of the cellular antioxidants defense system [20]. Therefore, it seems unlikely that Nrf2 plays a major role in the here observed

gene expression and no clear link between Nrf2 activity and IBC has been reported so far.

The relevance of MAPK signaling and AP-1 activation in IBC is unclear, but overexpression of AP-1 subunits such as c-Jun and MAPK hyperactivation have been suggested [66, 67]. While ROS-dependent TNC induction was independent of c-Jun, 83% of the CIH-upregulated genes could potentially be regulated by AP-1. Therefore, a contribution of AP-1 to CIH-dependent gene regulation in IBC cells cannot be excluded but would need further investigations to determine its relevance.

We also observed oxidative stress-dependent activation of the NF-κB signaling cascade at the level of or upstream of IκBα and TNC was regulated by activation of the NF-κB pathway. TNC has previously been suggested as a putative NF-κB target gene in IBC [60, 68], but although TNC is regulated by this transcription factor in other cell types, to our knowledge an NF-κB -dependent TNC regulation had not been shown in IBC cells before.

The term “inflammatory breast cancer” could suggest an inflammation-driven cancer, but it originated from the clinical appearance of patients [4, 69]. The role of inflammation in IBC aggressiveness is only beginning to be unraveled. Several studies indicated NF-κB to be constitutively activated in IBC and to be a major contributor to IBC-specific gene expression and tumorigenesis [2, 60, 67, 70]. As mechanisms for increased NF-κB activity, infection with human cytomegalovirus (HCMV), loss of estrogen receptor (ER) and differential cytokine expression has been suggested [2]. In our study, we found that oxidative stress activates NF-κB in IBC cells and that 64% of all CIH-upregulated genes can potentially be regulated or have been shown to be regulated by NF-κB, such as COX-2 [71]. TNC gene induction by oxidative stress was directly linked to NF-κB activation and increased TNC expression was associated with increased p105 expression in breast cancer tissue. Overall, these results demonstrate the importance of NF-κB in pro-metastatic gene regulation in IBC cells in response to intermittent hypoxia. Interestingly, TNC can activate toll-like receptor 4, which in turn activates NF-κB, leading to a regulatory loop [68, 72]. Furthermore, IH and inflammation can form a vicious circle *in vivo*, amplifying each other [73]. We therefore hypothesize that IH and oxidative stress play an important role in the constitutive activation of NF-κB in IBC, which is facilitated through increased TNC expression.

In summary, CIH is a possible novel modulator of IBC aggressiveness, increasing pro-metastatic gene expression through activation of NF-κB and maybe other transcription factors. Both CIH and NF-κB should be further investigated as potential targets for therapy of inflammatory breast cancer. The impact of CIH should be considered in future studies of IBC tumor material, as CIH affects gene and protein expression and could lead to differences in expression levels between different biopsy sites. Taking into consideration the crosstalk between the tumor microenvironment and the tumor, such as between intermittent hypoxia and IBC cells, might allow us to develop novel approaches for IBC treatment.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflicts of interest.

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FIGURE 1

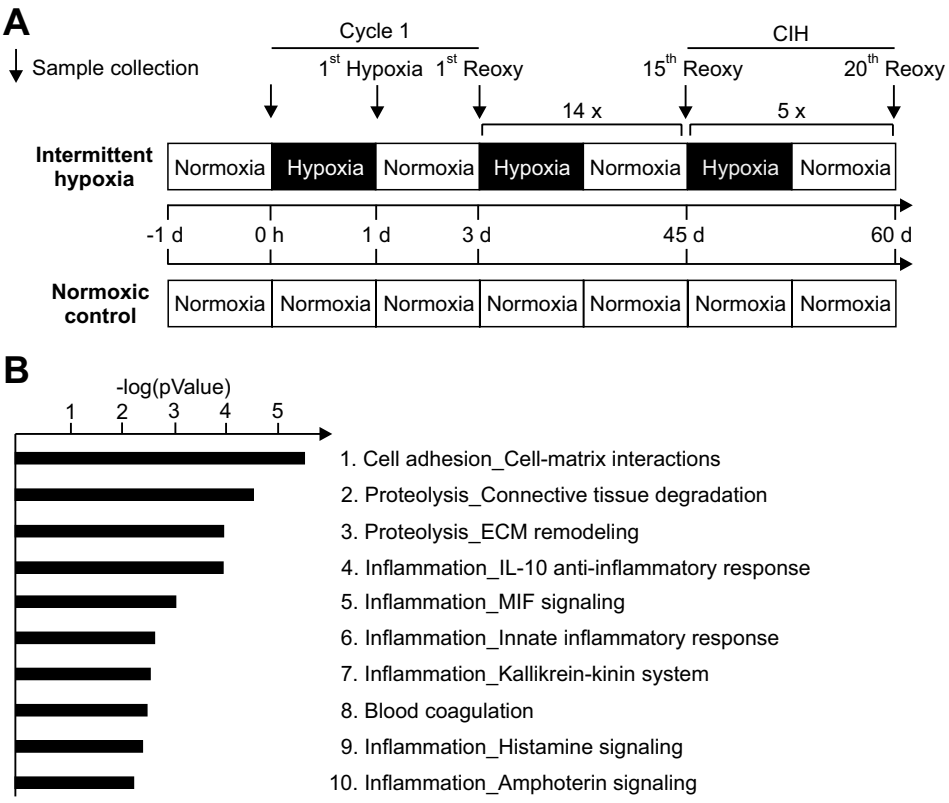


Fig. 1: Genes regulated by chronic intermittent hypoxia (CIH) in inflammatory breast cancer cells. (A) Experimental scheme of the CIH exposure regime, indicating the duration of exposure to hypoxia and normoxia, and the time points of sample collection. **(B)** Enrichment analysis of upregulated genes using MetaCore software to rank the most relevant cellular processes.

Figure 2

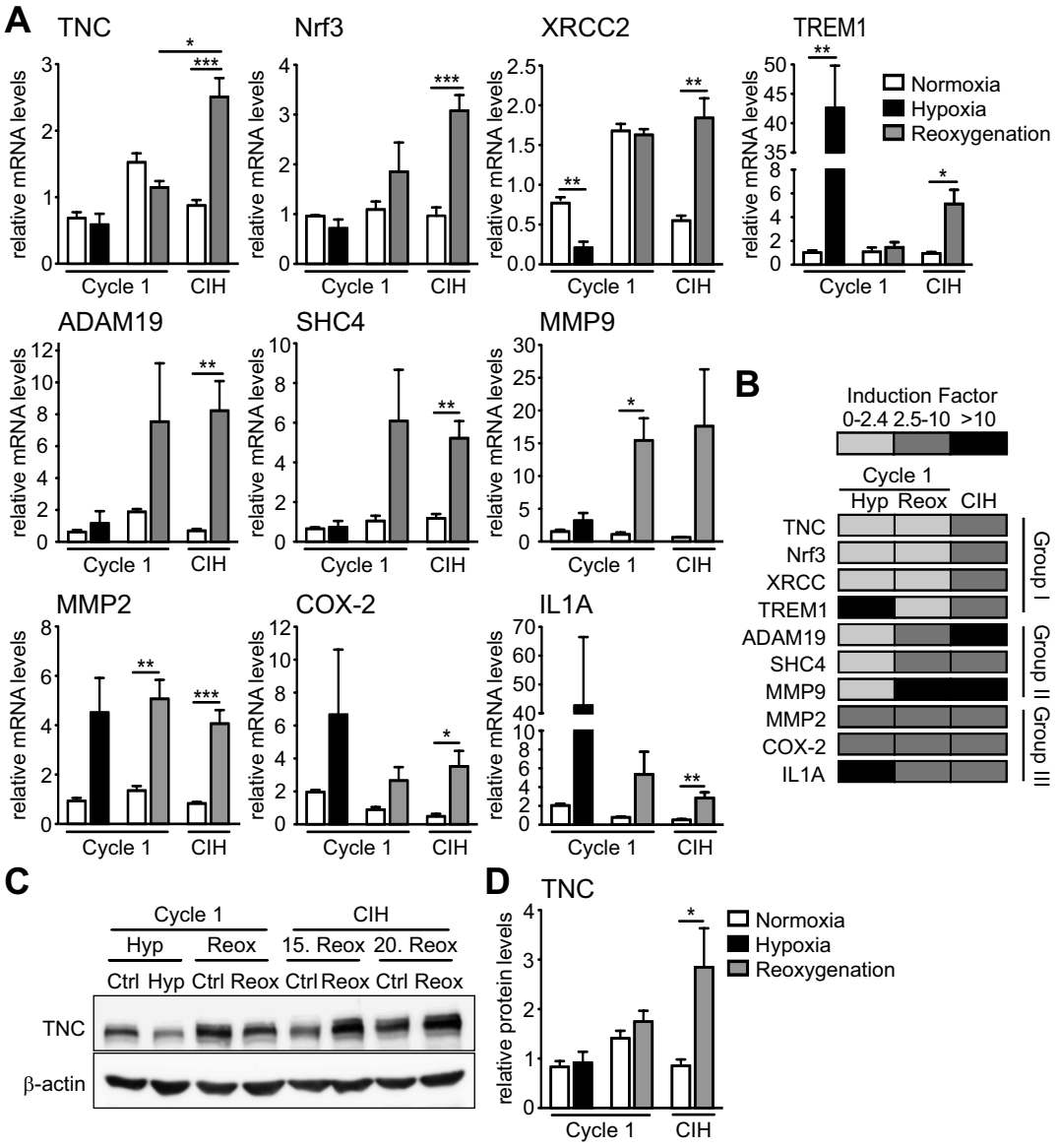


Fig. 2: Gene array validation reveals three distinct groups of genes regulated by CIH. (A) Validation of gene array results for selected candidate genes in SUM149PT cells by RT-qPCR. **(B)** Heatmap showing the mRNA induction factors depicted in (A) following hypoxia, reoxygenation or CIH. **(C)** Immunoblot analysis of TNC protein levels in response to hypoxia, reoxygenation or CIH. **(D)** Quantification of TNC protein levels following hypoxia, reoxygenation or CIH. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (Student's t-test).

Figure 3

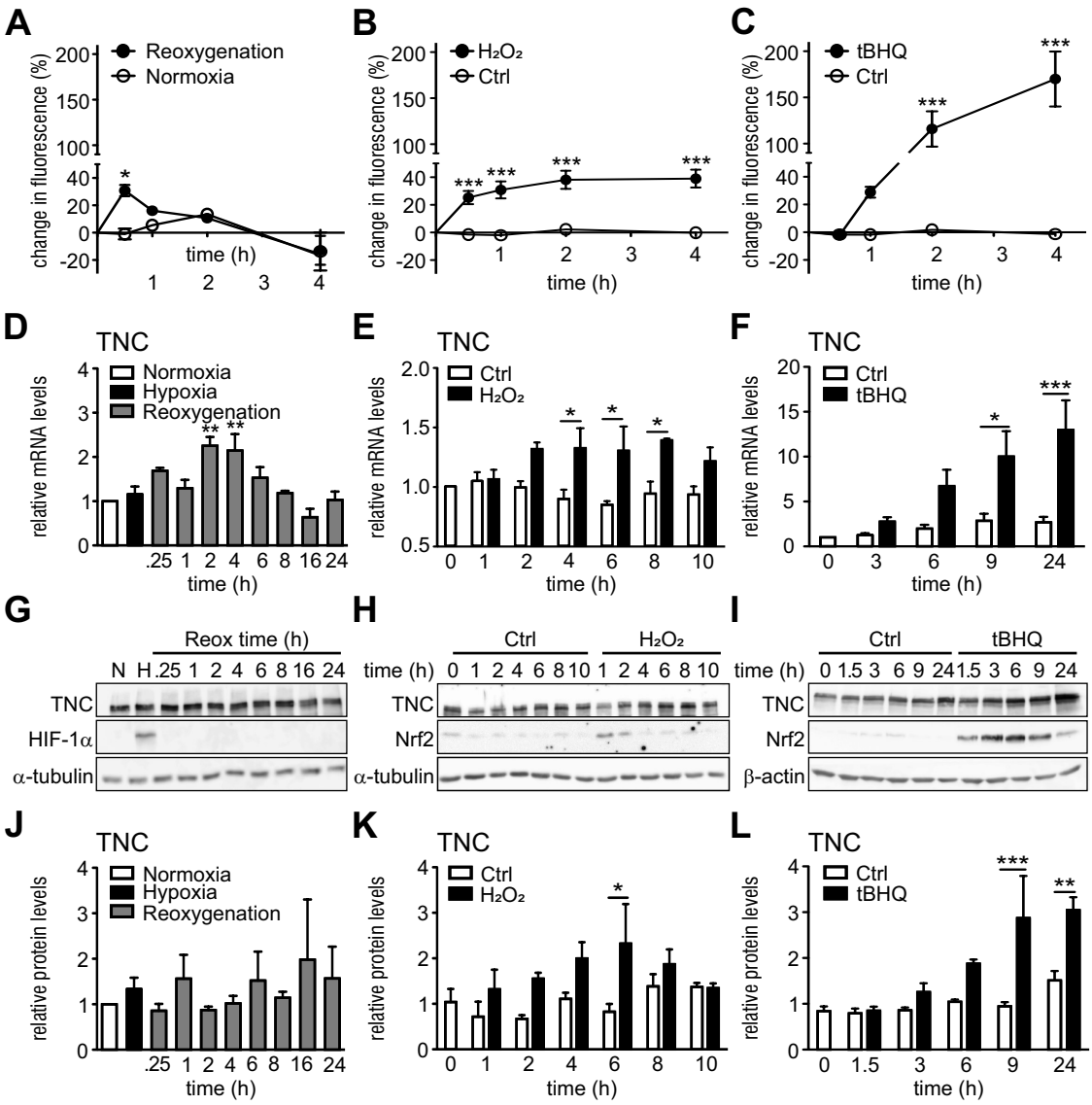


Fig. 3: Regulation of *TNC* gene expression by oxidative stress. H_2DCFDA assays to quantify the generation of intracellular ROS levels by **(A)** reoxygenation (following 24 h 0.2% O_2), **(B)** 100 μM H_2O_2 or **(C)** 30 μM tBHQ over 4 h. RT-qPCR analysis of TNC mRNA induction in response to **(D)** reoxygenation (following 24 h 0.2% O_2), **(E)** 100 μM H_2O_2 or **(F)** 30 μM tBHQ over the indicated time course. TNC immunoblot analysis in response to **(G)** reoxygenation (following 24 h 0.2% O_2), **(H)** 100 μM H_2O_2 or **(I)** 30 μM tBHQ over the indicated time course. Quantification of TNC protein levels in response to **(J)** reoxygenation (following 24 h 0.2% O_2), **(K)** 100 μM H_2O_2 or **(L)** 30 μM tBHQ over the indicated time course. *, $p<0.05$; **,

$p<0.01$; ***, $p<0.001$ (one-way ANOVA followed by Tukey test for (D, J); two-way ANOVA followed by Bonferroni posttest for all other statistical analyses).

Figure 4

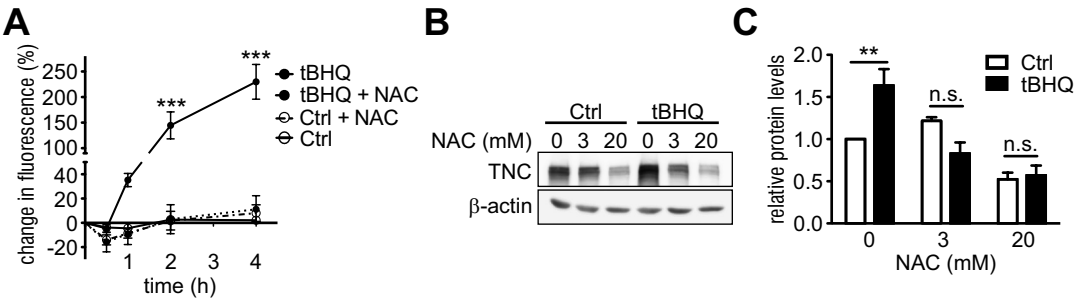


Fig. 4: Effects of antioxidants on tBHQ-mediated TNC induction. (A) H_2DCFDA analysis of tBHQ treatment (30 μM) alone or following 1 h pre-treatment with 3 mM NAC. **(B)** Analysis of TNC protein levels in SUM149PT cells in response to 30 μM tBHQ alone or following 1 h pre-treatment with 3 mM or 20 mM NAC. **(C)** Quantification of relative TNC protein levels. Values were normalized to β-actin loading control and to the starting time point. n.s., not significant; **, $p<0.01$; ***, $p<0.001$ (Two-way ANOVA followed by Bonferroni posttest was applied for comparison of tBHQ treatment alone with the combinatorial treatment of tBHQ and NAC in (A); one-way ANOVA followed by Tukey test in (C)).

Figure 5

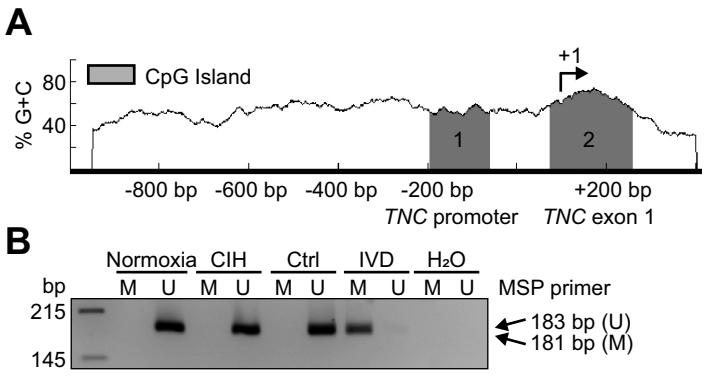


Fig. 5: Analysis of DNA methylation in the regulation of *TNC* expression by CIH. (A) Quantification of CpG density and CpG island location proximal to the transcriptional start site of the *TNC* gene with the MethPrimer software. **(B)** Methylation-specific PCR (MSP) targeting the CpG island in the *TNC* promoter. M, methylated DNA; U, unmethylated DNA; ctrl, control; CIH, chronic intermittent hypoxia; IVD, *in vitro* methylated DNA; bp, base pairs.

Figure 6

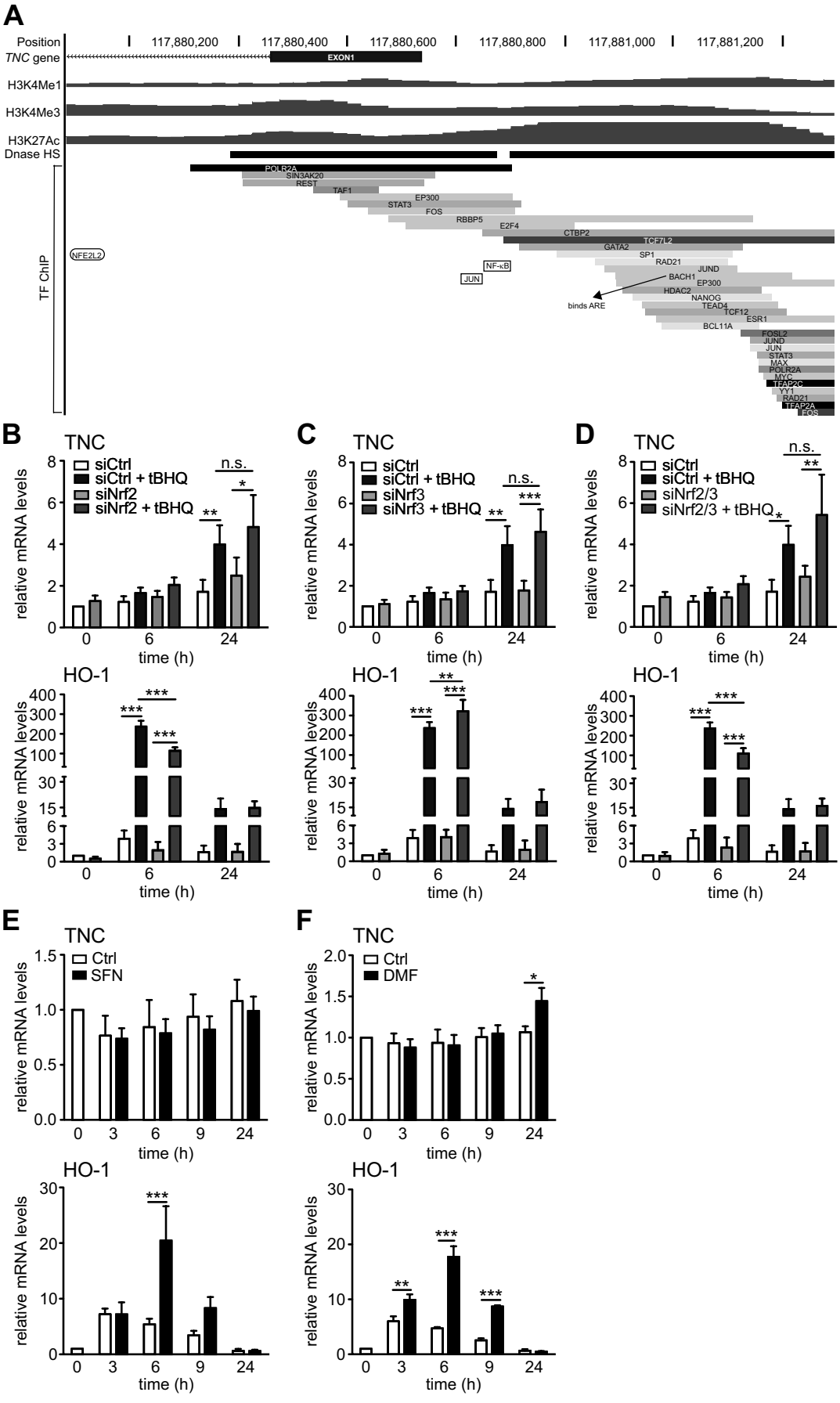

A
Position
117,880,200
117,880,400
117,880,600
117,880,800
117,881,000
117,881,200
TNC gene
EXON1
H3K4Me1
H3K4Me3
H3K27Ac
Dnase HS
TF ChIP
NFE2L2
NF-κB
JUN
binds ARE
B
TNC
siCtrl
siCtrl + tBHQ
siNrf2
siNrf2 + tBHQ
relative mRNA levels
time (h)
HO-1
C
siNrf3
siNrf3 + tBHQ
D
siNrf2/3
siNrf2/3 + tBHQ
n.s.
E
Ctrl
SFN
F
DMF

Fig. 6: Role of Nrf2 and Nrf3 in oxidative stress-dependent TNC regulation. (A) UCSC-integrated ENCODE data proximal to the transcriptional start site of the *TNC* gene. The transcription factor ChIP-sequencing track shows regions of transcription factor binding derived from ChIP-seq experiments performed by the ENCODE project. The intensity of the grey shading is proportional to the signal strength observed in any cell line contributing to the respective cluster. Enrichment levels of histone marks (H3K4Me1, H3K4Me3, H3K27Ac) were determined by ChIP-seq and indicate regions of active enhancers (H3K4Me1, H3K27Ac) or active promoters (H3K4Me3). DNase hypersensitive areas reflect regulatory regions. Further relevant TFBS' found in JASPAR and from published results were added in rectangles [65]. A circle shows relevant, additional TFBS' found in the Swissregulon database. JUN, c-Jun; ARE, antioxidant response element, targeted by Nrf2 and Nrf3. **(B)** Nrf2 or **(C)** Nrf3 alone or **(D)** Nrf2/Nrf3 in combination were knocked down for 48 h followed by treatment with 30 μM tBHQ or solvent control (ethanol) for the indicated time points. TNC, HO-1, Nrf2 and Nrf3 mRNA levels were determined by RT-qPCR. **(E)** Cells were treated with 10 μM SFN or solvent control (DMSO) and TNC and HO-1 mRNA levels were determined by RT-qPCR. **(F)** TNC and HO-1 mRNA levels were determined by RT-qPCR following treatment with 40 μM DMF or solvent control (DMSO). n.s., not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (two-way ANOVA followed by Bonferroni posttest).

Figure 7

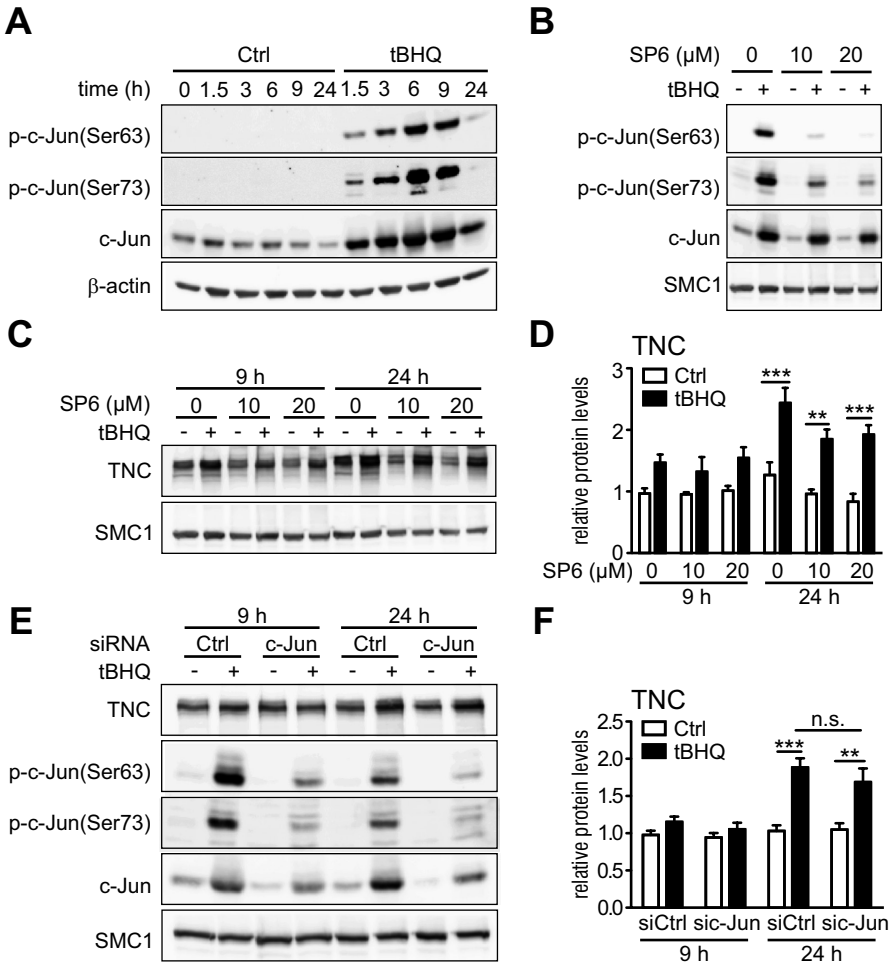


Fig. 7: Role of JNK and c-Jun activity in oxidative stress-dependent TNC regulation. (A) Immunoblot analysis of total c-Jun and phosphorylated c-Jun protein after treatment with 30 μM tBHQ or solvent control (ethanol) for the indicated time points. **(B)** SUM149PT cells were pre-treated for 1 h with different concentrations of the JNK inhibitor SP600125 (SP6) followed by a 9 h treatment with 30 μM tBHQ or solvent control (ethanol). Total c-Jun and phosphorylated c-Jun protein was detected by immunoblotting. **(C)** Immunoblot analysis of TNC protein expression in response to 30 μM tBHQ with or without 1 h pre-treatment with different concentrations of the JNK inhibitor SP600125 for the indicated time points. **(D)** Quantification of relative TNC protein expression levels. **(E)** Immunoblot analysis of TNC protein levels after knockdown of c-Jun for 48 h and followed by treatment with 30 μM tBHQ or solvent control (ethanol) for the indicated time points. **(F)** Quantification of relative TNC

protein expression levels. n.s., not significant; **, $p<0.01$; ***, $p<0.001$ (one-way ANOVA followed by Tukey test).

Figure 8

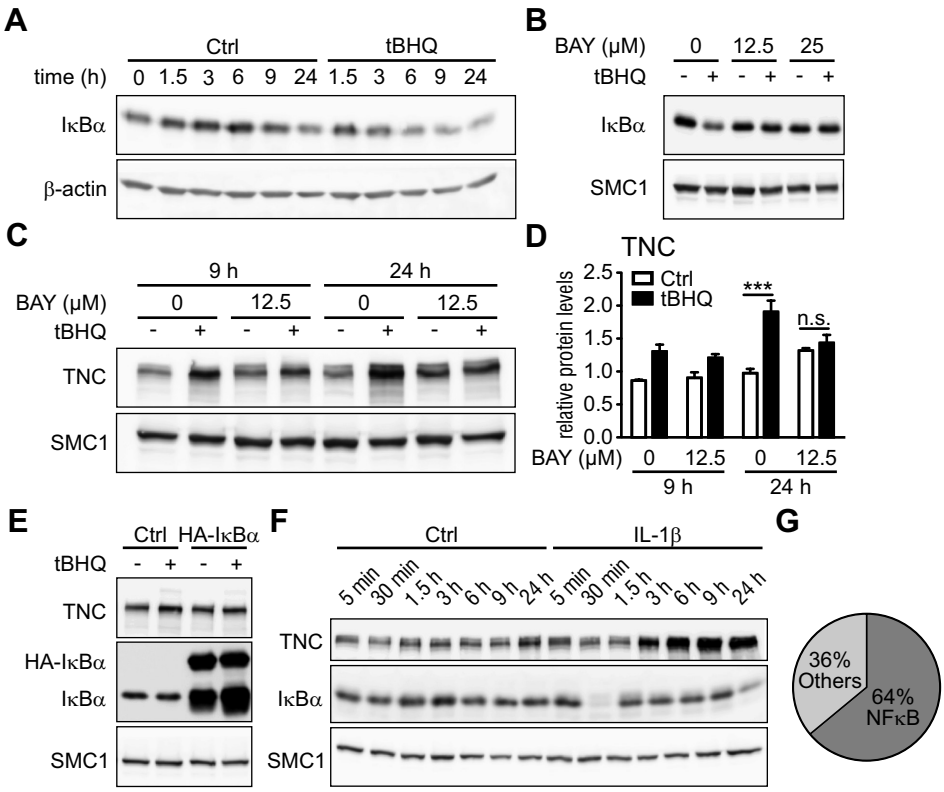


Fig. 8: Role of NF-κB in oxidative stress-dependent TNC regulation. (A) Immunoblot analysis of IκBα protein levels following treatment with 30 μM tBHQ or solvent control (ethanol) for the indicated time points. **(B)** IκBα protein levels were analysed by immunoblotting following 1 h pre-treatment with different concentrations of the IκB kinase (IKK) inhibitor BAY 11-7082 (BAY) and subsequent addition of 30 μM tBHQ or solvent control (ethanol) for 9 h. **(C)** SUM149PT cells were pretreated for 1 h with 12.5 μM BAY 11-7082 followed by 30 μM tBHQ or solvent control (ethanol) for indicated time periods. TNC protein levels were analysed by immunoblotting. **(D)** Quantification of TNC protein levels of the experiment depicted in (C). **(E)** Following transient overexpression of IκBα for 24 h, cells were treated with 30 μM tBHQ or solvent control (ethanol) for further 9 h and TNC protein levels were analyzed by immunoblotting. **(F)** SUM149PT cells were treated with 2 ng/ml IL-1β or solvent control (0.1% BSA in PBS) for the indicated time course and IκBα and TNC protein levels were analyzed by immunoblotting. **(G)** Analysis of NF-κB binding sites in all CIH-upregulated genes, observed in the gene array shown in Supplementary Table

S1. The graph depicts the combined results of TFBS identification using the web-based software oPOSSUM and MetaCore. oPOSSUM and MetaCore identified TFBS profiles based on JASPAR and published results, respectively. n.s., not significant; ***, $p<0.001$ (one-way ANOVA followed by Tukey test).

Figure 9

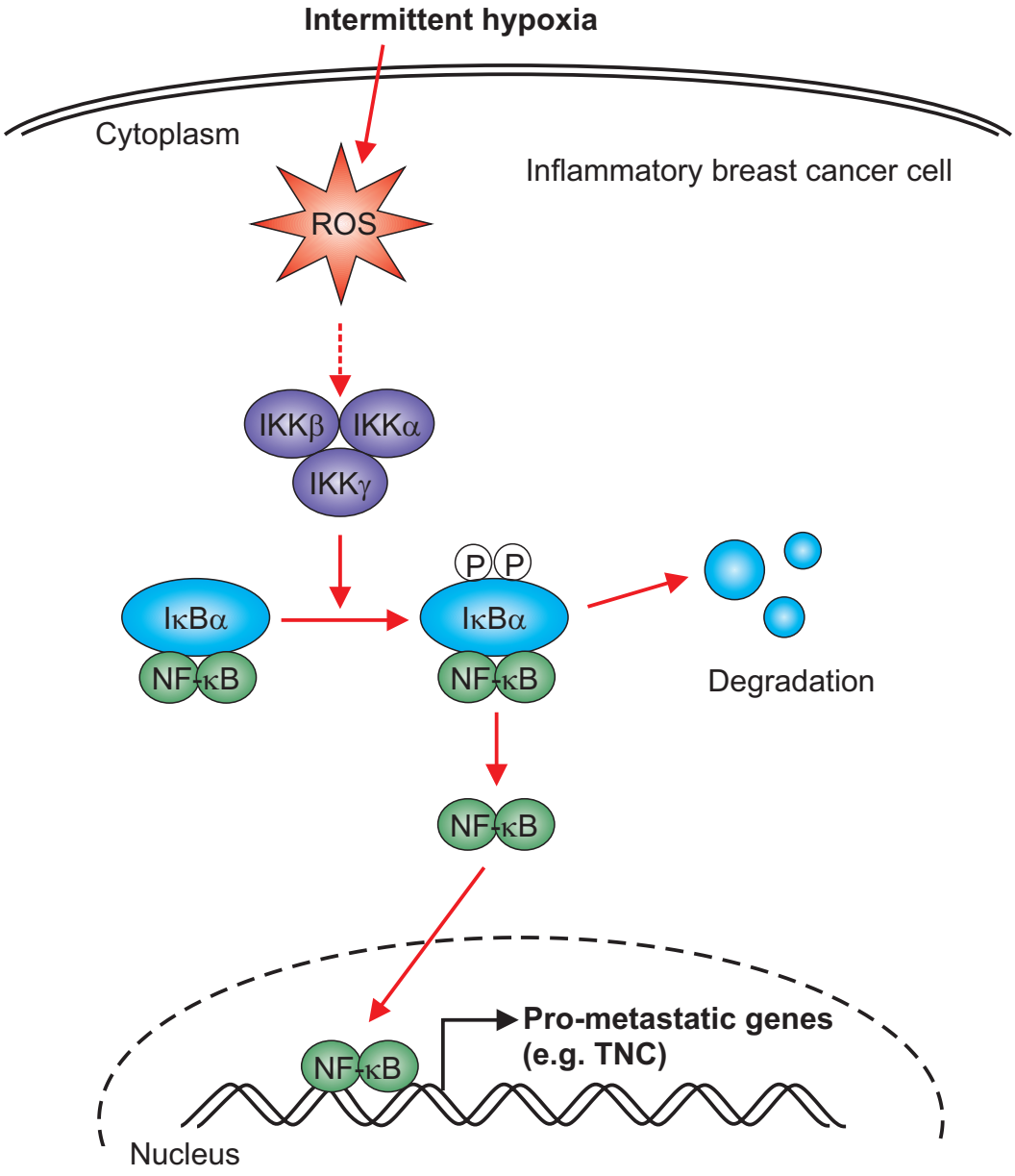
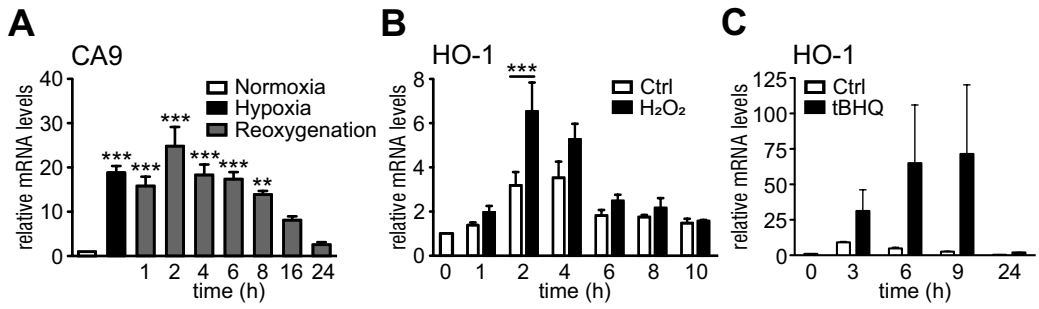


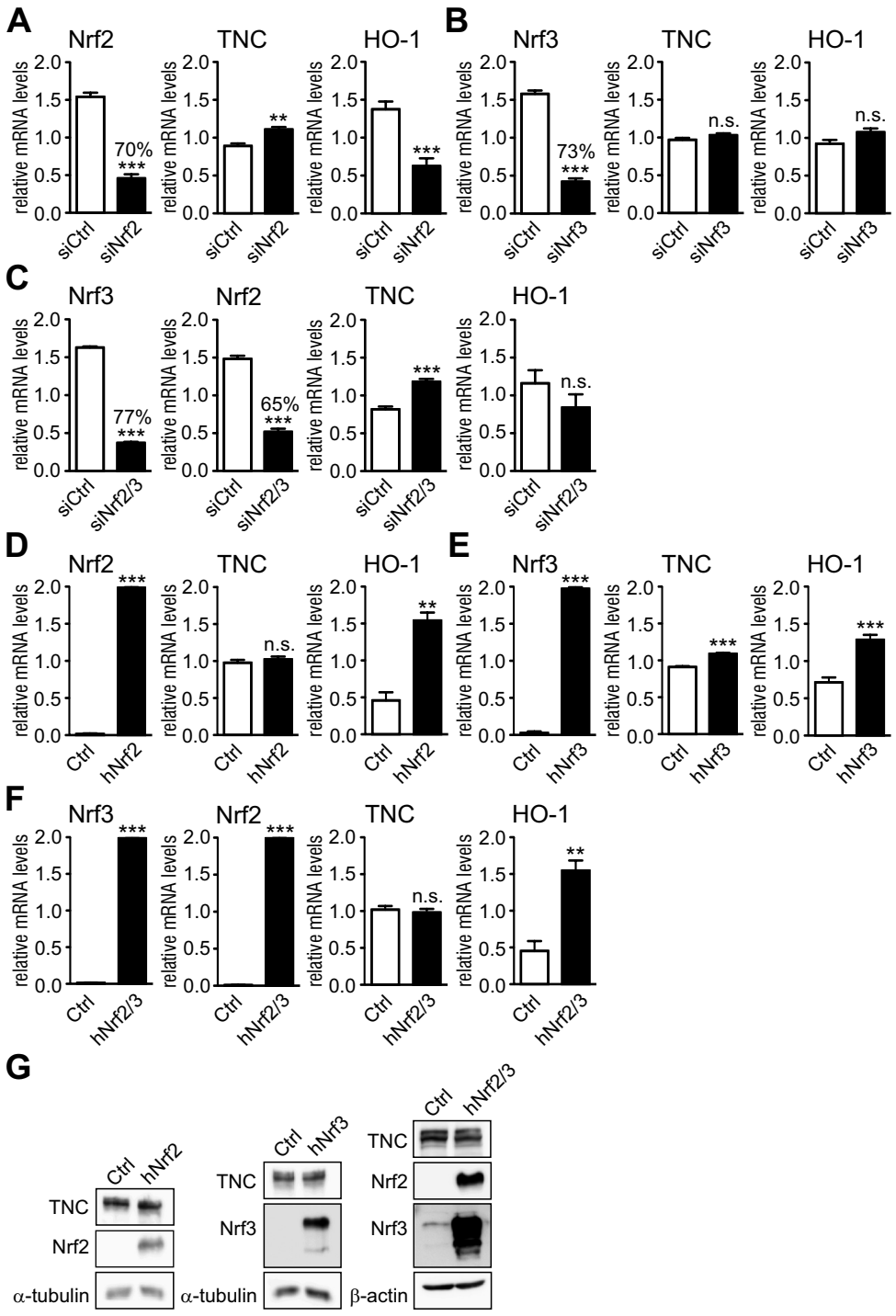
Fig. 9: Proposed model of the molecular mechanisms by which intermittent hypoxia mediates pro-metastatic gene expression via activation of NF-κB in inflammatory breast cancer cells. Intermittent hypoxia upregulates pro-metastatic genes in inflammatory breast cancer cells through activation of the transcription factor NF-κB.

Supplementary Figure 1



Supplementary Fig. S1: Hypoxia and ROS mediated regulation of a known HIF or Nrf2 target gene. (A) Samples shown in Fig. 3D (reoxygenation following 24 h of 0.2% O_2) were analysed for carbonic anhydrase IX (CA9) mRNA levels (a known HIF target gene) for the indicated time periods by RT-qPCR. **(B)** Analysis of heme oxygenase (HO-1) mRNA levels (a known Nrf2 target gene) in samples shown in Fig. 3E (100 mM H_2O_2 treatment) for the indicated time course by RT-qPCR. **(C)** Analysis of HO-1 mRNA levels in samples shown in Fig. 3F (30 mM tBHQ treatment) for the indicated time course by RT-qPCR. **, $p<0.01$; ***, $p<0.001$ (one-way ANOVA followed by Tukey test for comparisons of treatment to normoxic control in (A); two-way ANOVA followed by Bonferroni posttest to compare treatment with respective control for (B) and (C)).

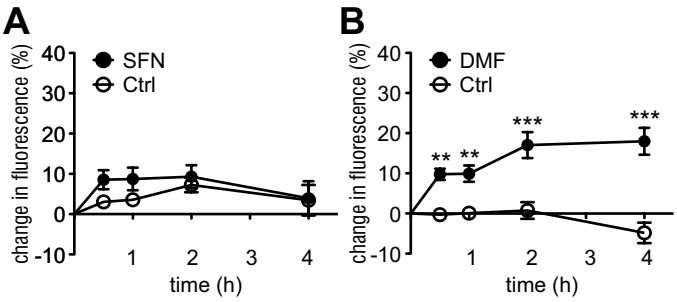
Supplementary Figure 2



Supplementary Fig. S2: Effects of single and simultaneous knockdown or overexpression of Nrf2 and Nrf3 on basal TNC expression. Knockdown of **(A)** Nrf2 or **(B)** Nrf3 alone or **(C)** in combination in SUM149PT cells for 48 h followed by RT-qPCR analysis of TNC, HO-1, Nrf2 and Nrf3 mRNA levels. Transient

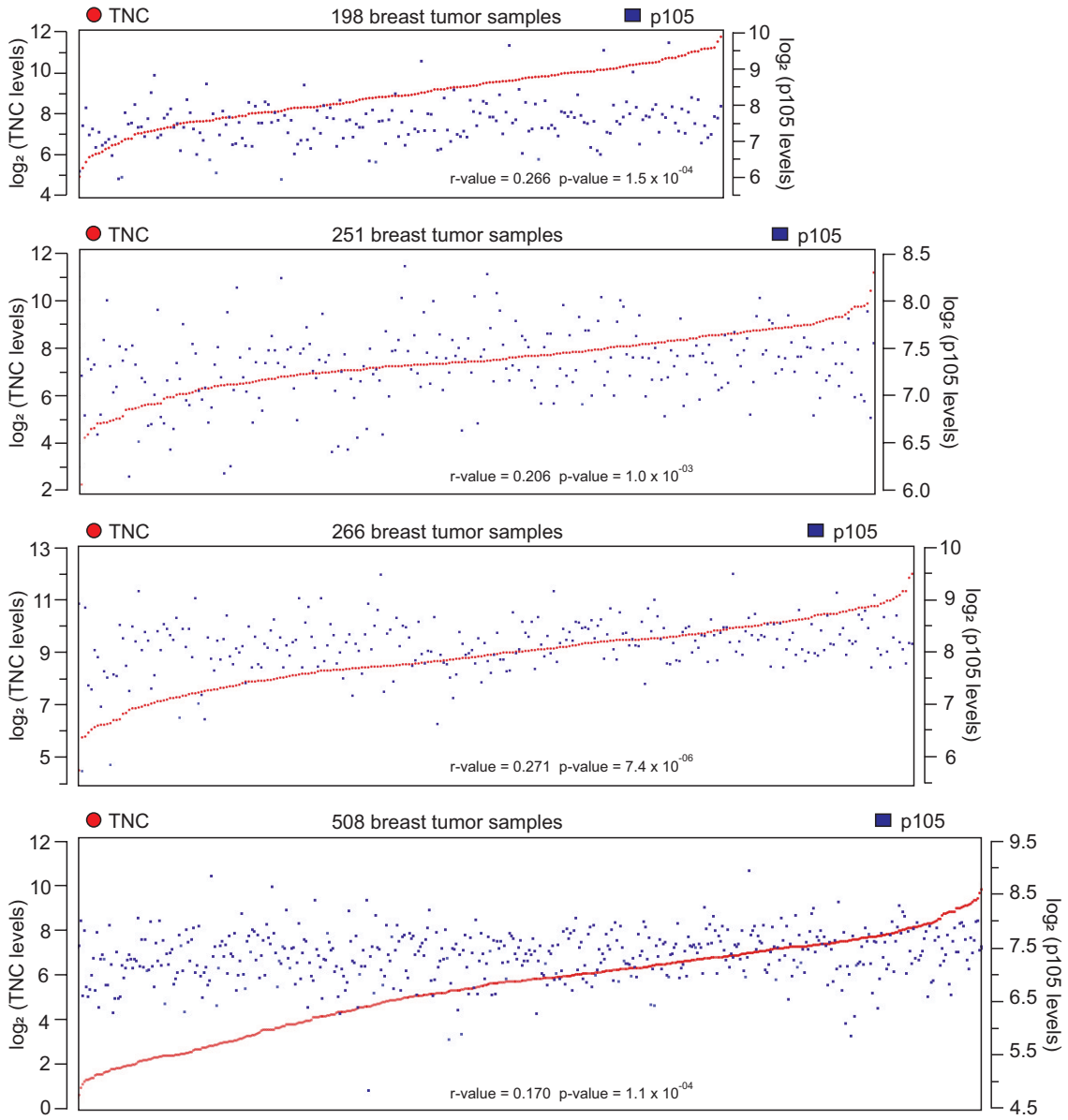
overexpression of **(D)** Nrf2 or **(E)** Nrf3 alone or **(F)** in combination for 24 h followed by RT-qPCR analysis of TNC, HO-1, Nrf2 and Nrf3 mRNA levels. U6 snRNA served as control gene and values were normalized to the average values of normoxic controls. **(G)** Immunoblot analysis for TNC, Nrf2 and Nrf3 in samples from cells with transient Nrf2 or Nrf3 overexpression alone or in combination. n.s., not significant; **, $p<0.01$; ***, $p<0.001$ (Student's t-test).

Supplementary Figure 3



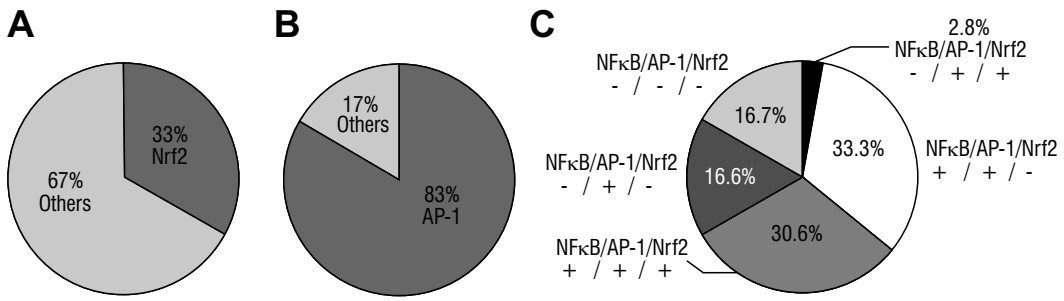
Supplementary Fig. S3: ROS production in SUM149PT cells following treatment with DMF or SFN. Quantification of ROS production with the H_2DCFDA assay in response to **(A)** 10 mM SFN or **(B)** 40 mM DMF. **, p<0.01; ***, p<0.001 (two-way ANOVA followed by Bonferroni posttest).

Supplementary Figure 4



Supplementary Fig. S4: Positive correlation between TNC and p105 expression in breast cancer transcriptomic data sets. Microarray data from four independent studies were compiled and analysed using the R2 genomic analysis tool. TNC mRNA levels positively correlate with p105 levels in breast cancer in all studies analysed, as assessed by one-way ANOVA.

Supplementary Figure 5



Supplementary Fig. S5: Analysis of TFBS' in CIH-upregulated genes for AP-1, Nrf2 and NF-kB. Upregulated genes of the gene array dataset shown in Fig. 1 were analysed for TFBS' using the web-based oPOSSUM and MetaCore softwares. The results of both analyses were combined and are shown as percentage of genes with TFBS' for **(A)** Nrf2 or **(B)** AP-1. **(C)** Genes with TFBS' for NF-kB, AP-1 and/or Nrf2 indicated by "+" for a TFBS being present and by "–" for a TFBS not having been found.

Supplementary Table S1: Genes upregulated by CIH in inflammatory breast cancer cells.

Gene Symbol	NCBI accession number	Gene description	Fold change	Matrisome Division	Category	Validated
MMP9	NM_004994	matrix metallopeptidase 9	51.59	Matrisome-associated	ECM Regulator	X
SPINK6	NM_205841, NM_001195290	serine peptidase inhibitor, Kazal type 6	16.67			
SERPINB2	NM_001143818, NM_002575	serpin peptidase inhibitor, clade B (ovalbumin), member 2	14.62	Matrisome-associated	ECM Regulator	
TREM1	NM_018643, NM_001242589, NM_001242590	triggering receptor expressed on myeloid cells 1	13.47			X
OLFML3	NM_020190	olfactomedin-like 3	12.00			
SERPINB10	NM_005024	serpin peptidase inhibitor, clade B (ovalbumin), member 10	10.73	Matrisome-associated	ECM Regulator	
ADAM19	NM_033274	ADAM metallopeptidase domain 19	8.19	Matrisome-associated	ECM Regulator	X
COL6A3	NM_004369, NM_057167, NM_057166, NM_057165, NM_057164	collagen, type VI, alpha 3	7.08	Core matrisome	Collagen	
SCARA5	NM_173833	scavenger receptor class A, member 5 (putative)	6.76			
NID2	NM_007361	nidogen 2 (osteonidogen)	6.30	Core matrisome	ECM Glycoprotein	
SHC4	NM_203349	SHC (Src homology 2 domain containing) family, member 4	6.20			X
KPNA7	NM_001145715	karyopherin alpha 7 (importin alpha 8)	6.10			
MMP2	NM_004530, NM_001127891	matrix metallopeptidase 2	5.79	Matrisome-associated	ECM Regulator	X
TNC	NM_002160	tenascin C	5.74	Core matrisome	ECM Glycoprotein	X
A2ML1	NM_144670	alpha-2-macroglobulin- like 1	5.70	Matrisome-associated	ECM Regulator	
ATP8A2	NM_016529	ATPase, aminophospholipid transporter, class I, type 8A, member 2	5.63			
ZNF750	NM_024702	zinc finger protein 750	5.57			
IL1A	NM_000575	interleukin 1, alpha	5.49	Matrisome-associated	Secreted Factor	X
FBN1	NM_000138	fibrillin 1	5.36	Core matrisome	ECM Glycoprotein	
IL1RN	NM_173842	interleukin 1 receptor antagonist	5.29	Matrisome-associated	Secreted Factor	
PTGS2	NM_000963	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	6.16			X
ODZ2	NM_001122679	odz, odd Oz/ten-m homolog 2	5.11			
GINS2	NM_016095	GINS complex subunit 2 (Psf2 homolog)	5.01			

Gene Symbol	NCBI accession number	Gene description	Fold change	Matrisome Division	Category	Validated
VCAN	NM_004385, NM_001164097, NM_001164098, NM_001126336	versican	4.97	Core matrisome	Proteoglycan	
NFE2L3	NM_004289	nuclear factor (erythroid- derived 2)-like 3	4.83			X
ALOX15B	NM_001141, NM_001039130, NM_001039131	arachidonate 15-lipoxygenase, type B	4.68			
PPP1R14C	NM_030949	protein phosphatase 1, regulatory (inhibitor) subunit 14C	4.57			
PLA2G4A	NM_024420	phospholipase A2, group IVA	4.54			
XRCC2	NM_005431	X-ray repair complementing defective repair in Chinese hamster cells 2	4.53			X
PTHLH	NM_002820, NM_198965, NM_198964, NM_198966	parathyroid hormone-like hormone	4.52			
SLC26A9	NM_052934, NM_134325	solute carrier family 26, member 9	4.52			
HS3ST3A1	NM_006042	heparan sulfate(glucosamine) 3-O-sulfotransferase 3A1	4.49			
KIF4B	NM_001099293	kinesin family member 4B	4.46			
CTSL2	NM_001333, NM_001201575	cathepsin L2	4.19			
ORC1	NM_004153, NM_001190818, NM_001190819	origin recognition complex, subunit 1	4.16			
POLQ	NM_199420	polymerase (DNA directed), theta	4.05			
CXorf49	NM_001145140	chromosome X open reading frame 49	4.04			

Listed are all at least four-fold upregulated genes in the gene array analysis following CIH in SUM149PT cells. Their indicated relation to the matrisome was based on analysis through the matrisome project (http://matrisomeproject.mit.edu/proteins/). RT-qPCR validated genes were indicated.

Supplementary Table S2: TFBS analysis by oPOSSUM and MetaCore for each CIH-upregulated gene.

	oPOSSUM analysis			MetaCore analysis			
Gene symbol	**RelA**	**c-Rel**	**p50**	**AP-1**	**Nrf2**	**RelA**	**c-Jun**
MMP9	X	X	X	X	X	X	X
SPINK6		X					
SERPINB2		X		X		X	X
TREM1		X		X		X	X
OLFML3	X	X		X	X		
SERPINB10				X			
ADAM19				X		X	
COL6A3	X			X	X		
SCARA5							
NID2	X	X	X	X			
SHC4		X		X			
KPNA7							
MMP2						X	X
TNC	X	X	X	X	X	X	X
A2ML1							
ATP8A2	X	X		X	X		
ZNF750				X			
IL1A				X		X	X
FBN1	X	X	X	X			
IL1RN	X	X		X		X	
PTGS2						X	X
ODZ2	X	X	X	X	X		
GINS2				X			
VCAN	X	X	X	X	X		X
NFE2L3	X	X	X	X			
ALOX15B				X	X		
PPP1R14C				X			
PLA2G4A						X	X
XRCC2							
PTHLH	X	X	X	X	X		
SLC26A9	X	X	X	X	X		
HS3ST3A1	X	X		X	X		
KIF4B							
CTSL2							
ORC1		X		X	X		
POLQ				X			

Analysis of TFBSs for all CIH-upregulated genes identified by gene array analysis in SUM149PT cells. oPOSSUM analysis identified TFBSs for NF-κB (subunits RelA, c-Rel and p50), AP-1 and Nrf2. MetaCore analysis identified genes that were reported to be transcriptionally regulated by RelA or c-Jun (subunit of AP-1) based on manually curated literature.

Supplementary Table S3: Primers used for RT-qPCR, MSP-PCR, or BSP-PCR.

RT-qPCR primers

U6 snRNA	forward	5'-CTCGCTTCGGCAGCACA-3'
	reverse	5'-AACGCTTCACGAATTTGCGT-3'
TNC	forward	5'-GAAGGTGGAGGGGTACAGTG-3'
	reverse	5'-TAACGCCCTGACTGTGGTTATT-3'
MMP2	forward	5'-CTCGCAAGCCCAAGTGGGACA-3'
	reverse	5'-CCATGCTCCCAGCGGCCAAA-3'
MMP9	forward	5'-CCCCAGCGAGAGACTCTACA-3'
	reverse	5'-CGGAGTAGGATTGGCCTTGG-3'
ADAM19	forward	5'-ACCTCGCAGGATGAAAAGGG-3'
	reverse	5'-CCGGATGTTCAAGGATCGGT-3'
SHC4	forward	5'-ACACTTAGGAGCAGGCAGGA-3'
	reverse	5'-TTGCTTCCCTTGTAACTTGGGT-3'
Nrf3	forward	5'-TGACTGGGAGGCAGAAAAGA-3'
	reverse	5'-TCAGGCTGTGATGAAAGCAAC-3'
HO-1	forward	5'-ATGACACCAAGGACCAGAGC-3'
	reverse	5'-GTGTAAGGACCCATCGGAGA-3'
CA9	forward	5'-ACCAGACAGTGATGCTGAGTG-3'
	reverse	5'-AAACCAGGGCTAGGATGTCAC-3'
Nrf2	forward	5'-GGATCTGCCAACTACTCCCAG-3'
	reverse	5'-GACTGAAACGTAGCCGAAGA-3'
IL1A	forward	5'-GATCAGTACCTCACGGCTGC-3'
	reverse	5'-GTGCCGTGAGTTTCCCAGAA-3'
TREM1	forward	5'-TCCTCCTACCACCACTAAGGC-3'
	reverse	5'-CATTCTCGTGGGTTCGTGGG-3'
COX-2	forward	5'-CAACTCTATATTGCTGGAACATGGA-3'
	reverse	5'-TGGAAGCCTGTGATACTTTCTGTACT-3'

MSP primers

TNC-methylated	forward	5'-TATAAGAGGGGAGTTAGGGTTGC-3'
	reverse	5'-AAACCCATTTACATACAATTTATAACGA-3'
TNC-unmethylated	forward	5'-AGTATAAGAGGGGAGTTAGGGTTGT-3'
	reverse	5'-AAACCCATTTACATACAATTTATAACAAA-3'

BSP primers

TNC promoter

forward	5'-TGTAAAACGACGGCCAGTTTTTTTTAGGAATTGGGTTTAG-3'
reverse	5'-CAGGAAACAGCTATGACCTTTCCCACTTTTTCAATTAACR-3'

TNC 1st exon

forward	5'-TGTAAAACGACGGCCAGATTGAAAAAGTGGGAAAGGAT-3'
reverse	5'-CAGGAAACAGCTATGACCCAACRATAAAAAAAAAAACCCC-3'

4. Unpublished data 1: Effect of chronic intermittent hypoxia-conditioning on the malignant properties of SUM149PT inflammatory breast cancer cells

INTRODUCTION

The oxygen levels in tumors are dynamic, with repeated cycles of hypoxia and reoxygenation, called intermittent hypoxia (IH). IH has been shown to promote tumor progression, metastasis and treatment resistance (Durand, 2001; Durand & Aquino-Parsons, 2001a; Martinive et al., 2006; Rofstad, Gaustad, Egeland, Mathiesen, & Galappathi, 2010). While inflammatory breast cancer (IBC) is rare, it is the most aggressive form of breast cancer (Anderson, Schairer, Chen, Hance, & Levine, 2005; Hance, Anderson, Devesa, Young, & Levine, 2005). The mechanisms leading to the aggressiveness of IBC are poorly understood. IBC does not occur as a single solid tumor, but is rather diffusely distributed and metastasizes rapidly (Silvera & Schneider, 2009; Yang et al., 2008). IBC is highly proliferative (Paradiso et al., 1989), highly angiogenic, and displays a higher intratumoral microvessel density than non-IBC tumors (Colpaert et al., 2003; McCarthy et al., 2002). Comparing gene expression between IBC and non-IBC revealed a more abundant expression of pro-angiogenic and hypoxia regulated genes in IBC, such as vascular endothelial growth factor A (*VEGFA*) and prostaglandin G/H synthase 2 (*PTGS2*/COX2) (Bieche et al., 2004). Furthermore, IBC tumor blood vessels are not fully developed (Vermeulen, van Golen, & Dirix, 2010). This suggests that IBC cells are exposed to IH. Silvera and Schneider further postulated that IBC cells have adapted to the hypoxic microenvironment by changing gene expression (Silvera & Schneider, 2009).

The role of IH in IBC and its impact on tumor progression is unclear. Therefore, we investigated the effect of chronic IH (CIH) on cancer cell malignant properties in the IBC cell line SUM149PT, performing different *in vitro* assays used to determine changes in the behavior of cancer cells.

MATERIAL AND METHODS

Cell culture and treatments

The human triple-negative breast cancer cell line SUM149PT was cultured in equal parts of high-glucose DMEM (Sigma-Aldrich) and Ham's F-12 Nutrient Mixture (Gibco, Thermo Fischer Scientific, Waltham, MA, USA), supplemented with 5% heat-

inactivated fetal bovine serum (FBS), 1 μg/ml hydrocortisone (Sigma-Aldrich), 5 μg/ml human insulin (Sigma-Aldrich), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich). H_2O_2 (Sigma-Aldrich) was diluted to the required concentration in DMEM/F-12 medium without FBS and supplements. Hypoxic experiments were carried out in a humidified atmosphere containing 0.2% O_2 and 5% CO_2 in a gas-controlled glove box (Invivo2 400, Baker Ruskinn, Bridgend, UK). For the CIH conditioning, cells were cultured for 20 cycles in CIH conditions (24 h 0.2% O_2, 48 h 21% O_2). In parallel, cells were cultured under standard normoxic conditions for the same duration of time. Scratch assays were performed directly following 20 cycles of CIH. For all other assays, CIH-conditioned cells have been frozen, were then thawed, and after some days of cell expansion, they were cultured again for 2-20 cycles in IH conditions before performing experiments.

Scratch assay

Cells were grown to full confluency in 6-well plates. Two straight lines were scratched with a 200 µl pipette tip, forming a cross. Microscopic images were taken directly and following 15 hours of cultivation under standard culture conditions. The cell-free area was measured using ImageJ 1.48v (http://imagej.nih.gov/ij) and converted to percentage scratch recovery.

Proliferation assay

To determine cell proliferation $8x10^4$ cells were seeded per well into 12-well dishes. Cells were cultivated in normoxia for up to 72 hours. Following 24, 48 and 72 hours cells were detached by trypsin/EDTA. Viable cells were counted via trypan blue exclusion using a Beckman Coulter Vi-cell XR Cell Viability Analyser (Brea, CA, USA).

Viability assays

To determine the impact of different stimuli on cell viability and cell proliferation, $1.6x10^5$ cells per well were seeded into 12-well dishes. For H_2O_2 experiments, cells were treated with different concentrations of H_2O_2 for 24 hours. For transient nutrient deprivation experiments, cells were cultivated in PBS for 6 hours, followed by cultivation in normal medium for 24 hours. For the comparison of normoxia and hypoxia, cells were cultivated for 48 hours in normoxia or hypoxia. Supernatant was

collected and cells were detached by trypsin/EDTA. Cell counting and determination of cell viability was performed via trypan blue exclusion using a Beckman Coulter Vi-cell XR Cell Viability Analyser.

Attachment assay

96-well plates were coated with Poly-L-Lysine (Sigma-Aldrich), collagen 1 (Sigma-Aldrich), fibronectin (Sigma-Aldrich), or left untreated. $2x10^4$ cells were seeded per well and incubated for 30 min under standard culture conditions. Following shaking and washing in order to remove non-attached cells, attached cells were fixed and stained with crystal violet.

H_2DCFDA assay

SUM149PT cells were seeded in 96-well plates at 80% confluency and incubated with 10 µM 2'7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Invitrogen) for 30 min in the dark. Subsequently, specific treatments were performed and fluorescence was measured using a 96-well fluorescence photometer (Infinite 200Pro, Tecan, Männedorf, Switzerland). Results were calculated as increase in fluorescence per well ((Ftx – Ft0) / Ft0*100), where Ftx = fluorescence at a certain time point and Ft0 = fluorescence at 0 min (Wang & Joseph, 1999). For hypoxia-reoxygenation experiments, 96-well plates were incubated for 24 h in 0.2% oxygen. Cells were incubated with H_2DCFDA inside the hypoxia workstation and fluorescence measurements were performed immediately thereafter.

Statistical analyses

Results are presented as mean values ± standard error of the mean (SEM). Statistical analyses were performed using one-way ANOVA followed by Tukey`s Multiple Comparison Test. P-values < 0.05 were considered statistically significant.

RESULTS

Effect of CIH on the migratory and proliferative potential of SUM149PT cells

To investigate the effect of CIH on tumor aggressiveness in IBC, the IBC cell line SUM149PT was cultured for 20 cycles in CIH conditions (24 h 0.2% O_2, 48 h 21% O_2). In parallel, SUM149PT cells were cultured under standard normoxic conditions (Ctrl). For subsequent assays, SUM149PT cells which were cultured only for a short

time period (Untreated) were included in order to determine changes that occurred due to long-term cultivation.

In order to analyze the effect of CIH on cell migration, scratch assays were performed. Scratch recovery occurred quickly compared to the ER-positive breast cancer cell line T47D (see Unpublished data 2) and neither CIH nor long-term cultivation caused changes in the migratory potential of SUM149PT cells (Figure 1A). Proliferation assays were performed in order to investigate if CIH led to enhanced cell divisions of SUM149PT cells. Over a time course of 72 hours, neither CIH nor long-term cultivation caused changes in the proliferative potential of SUM149PT cells (Figure 1B). Both assays were performed independently only twice, as we observed no differences.

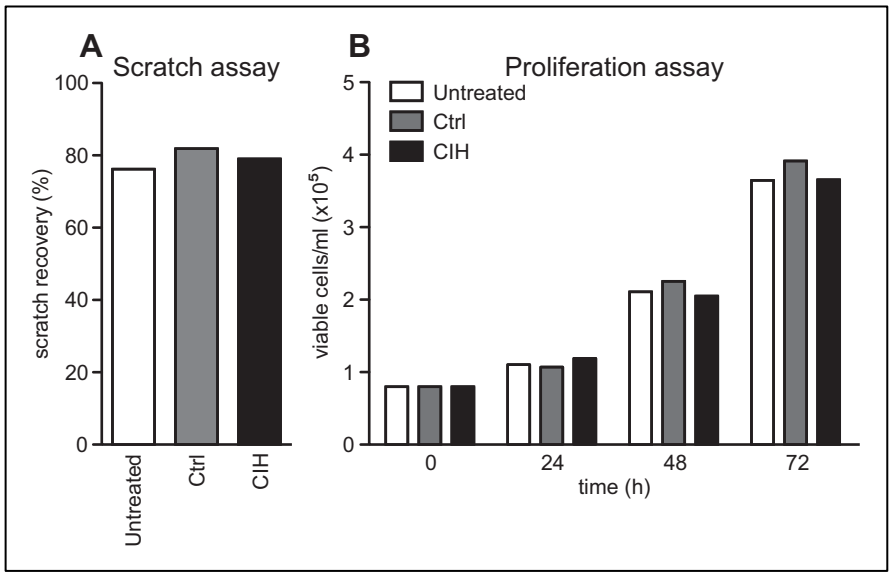


Figure 1: Effect of chronic intermittent hypoxia (CIH) on migratory and proliferative potential of SUM149PT cells. (A) Scratch assay to compare migratory potential of CIH-conditioned cells (CIH) with their respective normoxic control cells (Ctrl) and with cells that were only cultured for a short time period (Untreated) (n=2). **(B)** Proliferation of CIH-conditioned cells (CIH), their respective normoxic control cells (Ctrl) and cells that were cultured for a short time period (Untreated) (n=2).

Effect of transient nutrient deprivation, hypoxia and oxidative stress on cell viability and proliferation in CIH-conditioned and control cells

Under CIH conditions, tumor cells *in vivo* are exposed to hypoxia and/or oxidative stress. Moreover, due to the fluctuations in blood flow nutrient and growth factor delivery is insufficient and does not match the metabolic requirements. Therefore, we aimed to investigate if cells cultivated in CIH conditions *in vitro*, adapt to the before mentioned conditions.

Cells were treated for 24 hours with increasing concentrations of H_2O_2 as an oxidative stress stimulus. The effect on cell proliferation and cell viability was analyzed. 1 mM H_2O_2 caused a decrease in cell viability and was therefore toxic to SUM149PT cells (Figure 2A). H_2O_2 concentrations below 1 mM had no effect on cell viability but caused a decrease in the number of viable cells, hence showing an inhibitory effect on cell proliferation (Figure 2A). However, the observed effects were not statistically significant comparing CIH-conditioned cells with their respective normoxic controls and their short-term cultivation controls (Figure 2A).

In order to determine the effect of a transient nutrient deprivation in SUM149PT cells, cells were cultured for 6 hours in PBS, followed by cultivation in normal medium for 24 hours. Transient nutrient deprivation had no effect on cell viability, but cell proliferation was reduced by about 50% (Figure 2B). However, there was no statistically significant difference between CIH-conditioned cells, their respective normoxic controls and their short-term cultivation controls (Figure 2B).

With the purpose to compare cell proliferation and cell viability in normoxia and hypoxia, cells were cultivated for 48 hours in normoxic or hypoxic conditions. Cultivation in hypoxic conditions had no effect on viability or cell proliferation of SUM149PT cells (Figure 2C). Further, no difference could be observed between CIH-conditioned cells, their respective long-term cultivation control cells and their short-term cultivation control cells (Figure 2C).

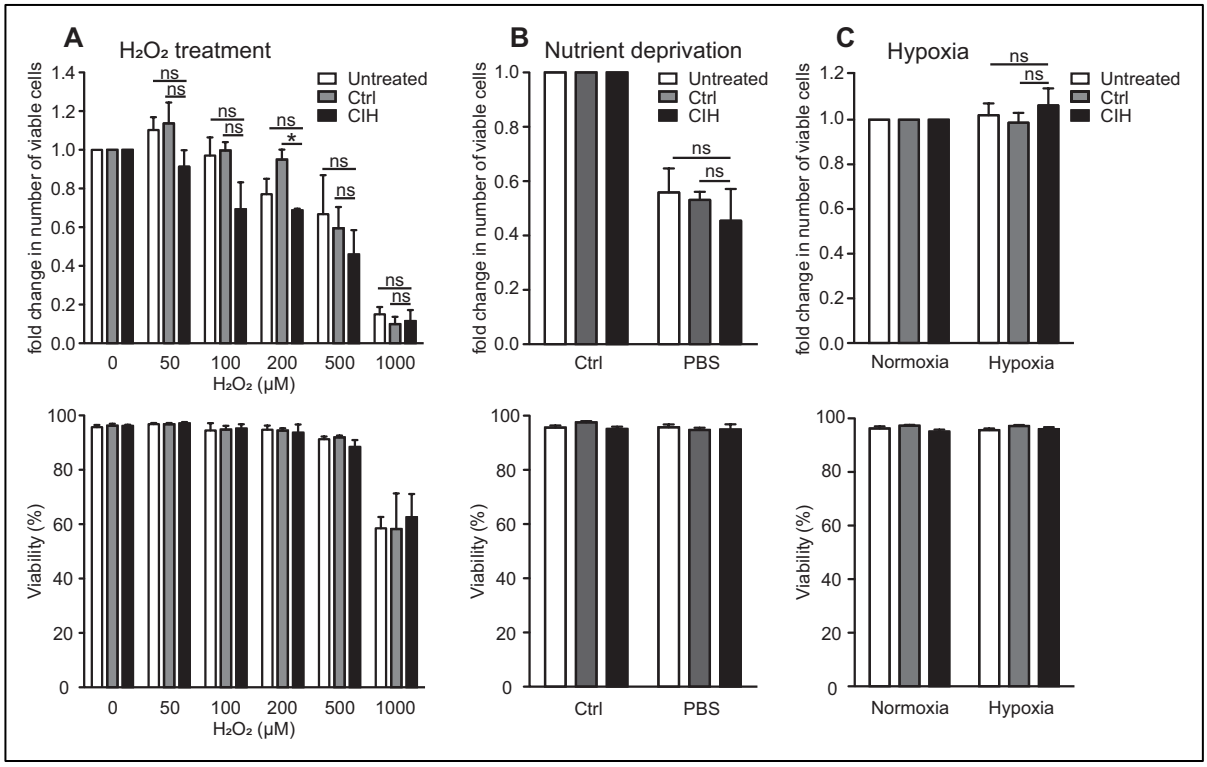


Figure 2: Effect of H_2O_2, nutrient deprivation or hypoxia on proliferation and viability in SUM149PT CIH-conditioned cells (CIH) their respective control cells (Ctrl) and short-term cultivated control cells (Untreated). (A) Cells were treated with different concentrations of H_2O_2 for 24 hours. Cell number and cell viability was assessed (n=3). ns, not significant; *p<0.05. **(B)** Cells were cultured for 6 hours in PBS, followed by cultivation in normal medium for 24 hours. Cell number and cell viability was assessed (n=3). ns, not significant. **(C)** Cells were cultured for 48 hours in normoxic or hypoxic conditions. Cell number and cell viability was assessed (n=3). ns, not significant.

Effect of CIH on stimuli-dependent intracellular ROS generation

Chronic intermittent hypoxia is widely considered as a source of intracellular reactive oxygen species (ROS) (Dewhirst, Cao, & Moeller, 2008; Nanduri, Yuan, Kumar, Semenza, & Prabhakar, 2008). Therefore, we aimed to assess if CIH-conditioned cells have adapted to ROS. H_2DCFDA assays were performed in order to determine the level of ROS generation during 30 min of reoxygenation (following 24 hours in hypoxia) in SUM149PT cells. Cells were also treated with 100 µM H_2O_2 and the change in DCF fluorescence during 30 min of treatment was determined. Further, the reoxygenation stimulus was combined with the H_2O_2 treatment. All treatments caused an increase in DCF fluorescence. While H_2O_2 treatment had the lowest effect, the combined treatment of H_2O_2 and reoxygenation showed the highest increase in DCF fluorescence (Figure 3). Comparing the response of CIH-

conditioned cells with the response of their respective control cells and the response of the short-term cultivated cells, no significant difference was detected (Figure 3).

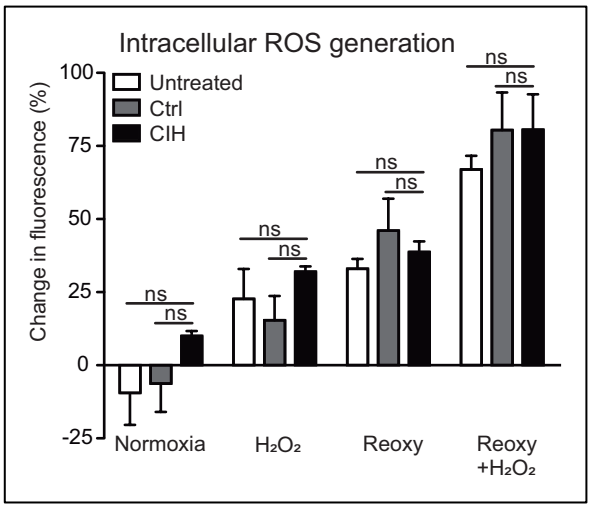


Figure 3: Comparison of stimuli-dependent intracellular ROS generation in CIH-conditioned cells (CIH), their respective control cells (Ctrl) and short-term cultivated control cells (Untreated). H_2DCFDA assays were performed to quantify the generation of intracellular ROS levels by treatment with 100 µM H_2O_2, upon reoxygenation and upon combined reoxygenation and treatment with 100 µM H_2O_2 over 30 minutes (n=3-4). ns, not significant.

Effect of CIH on cell attachment

We observed a CIH-mediated upregulation of genes encoding for extracellular matrix (ECM) proteins (see chapter 3: Manuscript). This might mediate differential attachment properties of SUM149PT cells towards different ECM components. Therefore, attachment assays were performed in order to investigate the cell attachment to collagen 1 and fibronectin. Cells were seeded on coated cell culture dishes and allowed to attach for 30 minutes. Shaking and washing removed non-attached cells. Poly-L-lysine is a nonspecific attachment factor that promotes cell attachment by enhancing the electrostatic interaction between negatively charged ions of the cell membrane and the culture dish surface and was therefore used for normalization of the results observed for attachment to collagen 1 and fibronectin. The highest level of cell attachment occurred with collagen 1, while there was no difference between CIH-conditioned and control cells (Figure 4). Investigating the attachment to fibronectin, CIH-conditioned cells showed the highest level of attachment, which was significantly different compared to short-term cultured control cells. However, compared to their respective long-term cultured control cells, the difference was not significant (Figure 4).

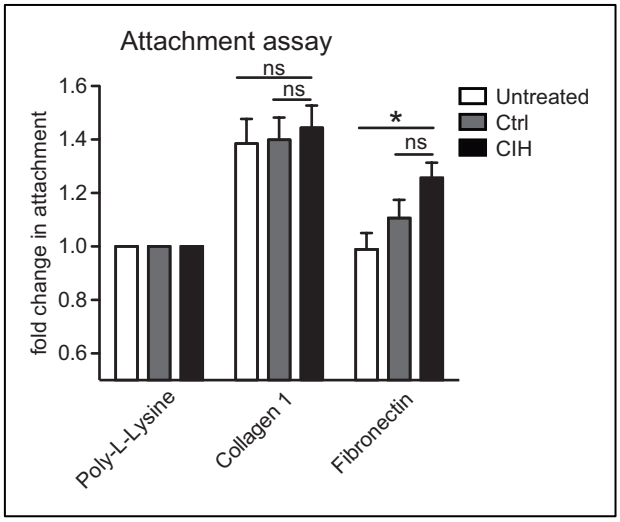


Figure 4: Comparison of attachment to different substrates of CIH-conditioned cells (CIH), their respective control cells (Ctrl) and short-term cultivated control cells (Untreated). Cell attachment assays were performed to determine the level of cell attachment to poly-L-lysine, collagen 1 or fibronectin (n=4). *p<0.05; ns, not significant.

DISCUSSION

CIH has been linked to tumor progression and tumor aggressiveness in various tumor models (Durand & Aquino-Parsons, 2001b; Rofstad et al., 2010). However, so far the impact of CIH on tumor progression in inflammatory breast cancer has not been investigated. Here, we studied the effect of CIH on cancer cell malignant properties in the inflammatory breast cancer cell line SUM149PT. Gene expression analysis revealed an enhanced expression of pro-metastatic genes following CIH (see chapter 3: Manuscript). This could suggest that these changes transfer into a more aggressive phenotype of SUM149PT cells. Therefore, various *in vitro* assays were performed in order to determine if CIH changes the malignant properties of SUM149PT cells.

We could detect a trend towards a differential attachment behavior upon CIH, as observed on fibronectin-coated dishes. This is in accordance with the identified enhanced expression of ECM proteins, suggesting ECM remodeling and hence differential attachment characteristics. Here, we only studied the attachment to collagen I and fibronectin. For further evaluation of the attachment characteristics the assay should be extended with additional ECM components as coating substrates, such as laminin or fibrillin, or with a combination of different substrates.

In all other performed assays we did not observe significant CIH-mediated changes in the malignant properties of SUM149PT cells. There are different factors to

consider while interpreting these results. The first point to take into consideration is that only the scratch assay was performed directly following 20 cycles of CIH and therefore also in close time proximity to the sample collection for gene expression analyses (see chapter 3: Manuscript). For all other experiments, CIH-conditioned cells have been first frozen, were then thawed, and after some days of cell expansion they were cultured again in IH conditions before performing experiments. Even though the cells were cultured for at least two cycles in IH conditions before performing further experiments, the interrupted cultivation and especially the process of freezing and thawing, might have reversed the observed CIH-mediated changes in gene expression and the potential behavioral changes. The second point to consider is that IBC is the most aggressive and most lethal form of breast cancer (Fouad, Kogawa, Reuben, & Ueno, 2014). SUM149PT cells were isolated from a patient with triple negative IBC. Hence, these cells host already very aggressive features, which we also observed with the performed *in vitro* assays. Scratch recovery in a scratch assay occurred very rapidly compared to T47D cells (see chapter 5: Unpublished data 2). Furthermore, SUM149PT cells showed a resistance to high levels of oxidative stress, whereby only a H_2O_2 concentration of 1 mM showed a toxic effect. Further, neither transient nutrient deprivation nor hypoxia had a negative effect on cell viability. Cultivation in hypoxia for two days did not even inhibit cell proliferation. This is in accordance with the findings of Silvera and Schneider (Silvera & Schneider, 2009) who exposed SUM149PT cells to hypoxia for 24 hours and found them to be highly resistant to protein synthesis inhibition, especially in comparison with the non-transformed MCF10A cells. Considering this highly aggressive phenotype of SUM149PT cells, it is reasonable to assume that CIH did not cause a major additional enhancement in aggressiveness, which could have been detected with the *in vitro* assays performed herein. *In vivo* assays might give different results. This was e.g. observed by Herrmann et al. (Herrmann et al., 2015), where hypoxic preconditioning of neuroblastoma cells caused a more aggressive phenotype *in vivo*, while *in vitro* no differences could be detected. In order to analyze the impact of CIH on mediating phenotypic changes relevant for tumor progression, it should be further considered to use a non-tumorigenic cell line, like the spontaneously immortalized human breast epithelial cell line MCF-10A (Soule et al., 1990).

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5. Unpublished data 2: Effect of chronic intermittent hypoxia on gene expression and cancer cell malignant properties in T47D cells

INTRODUCTION

In the previous chapters, we analyzed the effect of chronic intermittent hypoxia (CIH) on gene expression and cancer cell behavior in the triple-negative inflammatory breast cancer (IBC) cell line SUM149PT. Here, we aimed to study the impact of CIH on transcriptional regulation and cancer cell malignant properties in a non-IBC cell line, and selected the estrogen receptor-positive breast cancer cell line T47D.

MATERIAL AND METHODS

Cell culture and treatments

The human breast cancer cell line T47D was cultured in high-glucose DMEM (Sigma-Aldrich), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich). Hypoxic experiments were carried out in a humidified atmosphere containing 0.2% O_2 and 5% CO_2 in a gas-controlled glove box (Invivo2 400, Baker Ruskinn, Bridgend, UK). For the CIH experiment, cells were cultured for 20 cycles in CIH conditions (24 h 0.2% O_2). In parallel, cells were cultured under standard normoxic conditions for the same duration of time.

mRNA analysis and data deposition

The RNeasy mini kit (Qiagen, Hilden, Germany) was used for total RNA extraction according to manufacturer's protocol. Affymetrix Human Gene 2.1 ST strip arrays were used by the Functional Genomics Center Zurich (FGCZ) to analyze transcript levels of one biological replicate of untreated cells and of cells cultured for 60 days in CIH. For validation of the Affymetrix gene array results, three biological replicates of untreated cells and cells that were cultured for 60 days in normoxia or CIH conditions were analyzed by RT-qPCR. cDNA synthesis was based on 1 µg RNA using M-MLV Reverse Transcriptase (Promega Corporation, Madison, WI, USA) according to Manufacturer's recommendation. Reverse transcription-quantitative PCR (RT-qPCR) was performed with Roche SYBR Green 1 Master (Roche Diagnostics Ltd., Rotkreuz, Switzerland) and samples were analyzed on the LightCycler480 Instrument II (Roche Diagnostics Ltd.). The relative mRNA expression was quantified with the

LightCycler480 quantification software. U6 snRNA served as control gene and values were normalized to the average values of normoxic controls. Primer sequences are listed below:

Gene name	Forward primer (5`-3`)	Reverse primer (5`-3´)
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
STC1	AGTGGCGGCTCAAAACTCA	TGACGAATGCTTTTCCCTGA
SULF1	ACTGTACCCCAATGCTTCCC	GCATGTTATACAGCCTCTCCAC

Scratch assay

Cells were grown to full confluency in 6-well plates. Two straight lines were scratched with a 200 µl pipette tip, forming a cross. Microscopic images were taken directly and following 24 and 48 hours of cultivation under standard culture conditions. The cell-free area was measured using ImageJ 1.48v (http://imagej.nih.gov/ij) and converted to percentage scratch recovery.

Proliferation assay

To determine cell proliferation, cells were cultivated in normoxia for up to 72 hours. Following 24, 48 and 72 hours, the number of viable cells was estimated by using an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolim) assay (Cell Titer Aqueous, Promega, Madison, WI, USA) according to the manufacturer's protocol.

Statistical analyses

If not otherwise indicated, results are presented as mean values ± standard error of the mean (SEM). Statistical analyses were performed using Student's t-test or one-way ANOVA followed by Tukey's Multiple Comparison Test where appropriate. P-values < 0.05 were considered statistically significant.

RESULTS

Effect of CIH on gene expression in T47D cells

In order to investigate the effect of CIH on gene expression and cancer cell behavior in a non-IBC cell line, the human estrogen receptor-positive breast cancer cell line T47D was cultured for 20 cycles in CIH conditions (24 h 0.2% O_2, 48 h 21% O_2). As control, T47D cells were cultured in parallel under standard normoxic conditions

(Figure 1). Gene expression of untreated cells and cells that were cultured in CIH conditions for 60 days was assessed by whole genome microarray hybridization. 53 genes showed to be more than 2-fold upregulated following CIH with the highest fold change of 8. 20 genes showed to be more than 2-fold downregulated following CIH with the highest negative fold change of 4.

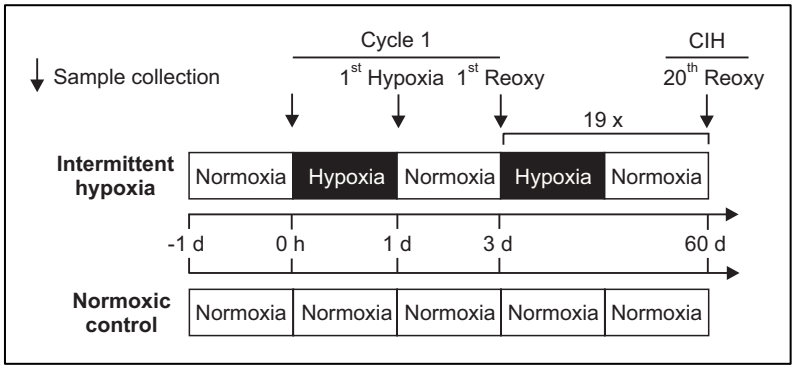

Figure 1: Experimental scheme of the CIH time schedule, indicating the duration of exposure to hypoxia and normoxia, and the time points of sample collection.

The CIH experiment was repeated to validate the expression levels of one up- and one down-regulated candidate gene, which were chosen because they are known to play a possible role in tumor progression. Expression of stanniocalcin 1 (STC1) showed to be 3-fold upregulated following CIH. *STC1* encodes for a secreted glycoprotein and has been linked to tumor progression. High STC1 expression in tumor samples has been correlated to a poor prognostic outcome (McCudden, Majewski, Chakrabarti, & Wagner, 2004). STC1 overexpressing cells showed enhanced proliferation and migration (Liu et al., 2010). Expression of sulfatase 1 (SULF1) was 2-fold downregulated following CIH. *SULF1* encodes for an extracellular heparan sulfate endosulfatase. SULF1 selectively removes 6-O-sulfate groups from heparan sulfate chains of heparan sulfate proteoglycans (HSPGs), a key constituent of the extracellular matrix (Morimoto-Tomita, Uchimura, Werb, Hemmerich, & Rosen, 2002). SULF1 expression is decreased in multiple malignant lineages, and its re-expression is known to be associated with decreased signaling of heparin-binding growth factors, cell proliferation and the invasiveness of cancer cells (Abiatari et al., 2006; Lai et al., 2003; Lai et al., 2004).

Gene expression of these genes was analyzed in response to hypoxia, reoxygenation (both part of cycle 1 of the CIH experiment; Figure 1) and CIH (20 cycles; Figure 1). STC1 and SULF1 showed no significant regulation in response to

hypoxia or in response to a single reoxygenation stimulus, but following 20 cycles of CIH, STC1 was significantly upregulated (Figure 2A) and SULF1 was significantly downregulated (Figure 2B).

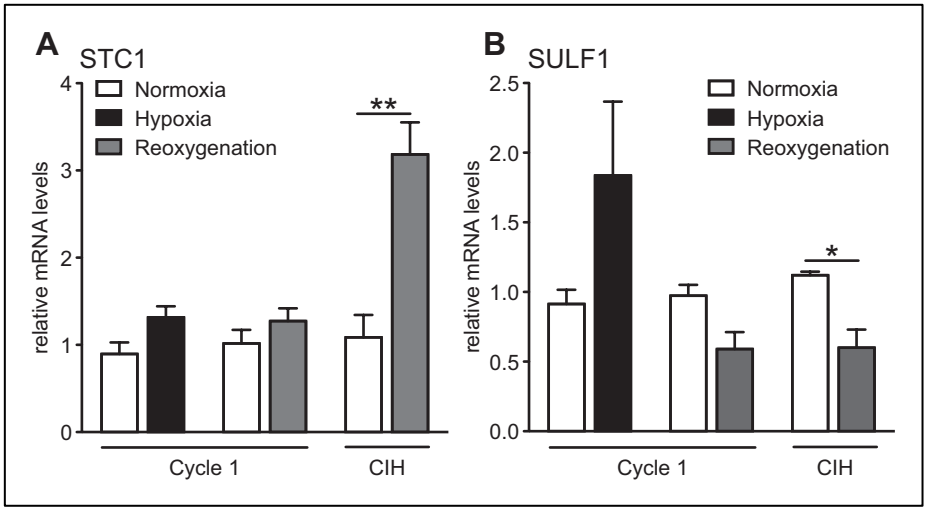

Figure 2: Gene array validation identifies genes regulated by CIH. (A+B) Validation of gene array results for selected candidate genes in T47D cells by RT-qPCR (n=3). *p<0.05; **p<0.01; Student's t-test.

Effect of CIH on migratory and proliferative potential of T47D cells

Scratch and proliferation assays were performed to compare the phenotype of cells that were cultured in CIH conditions with their respective normoxic controls (Ctrl). Comparisons were also carried out with T47D cells that were cultured only for a short time period (Untreated) in order to determine changes that occurred due to long-term cultivation.

Scratch assays were performed in order to analyze the effect of CIH on cell migration. Scratch recovery occurred slowly compared to SUM149PT cells (see chapter 4: Unpublished data 1). After 48 hours, scratches recovered by 70% and neither CIH nor long-term cultivation caused changes in the migratory potential of T47D cells (Figure 3A). Proliferation assays were performed in order to investigate the impact of CIH on cell proliferation. Only one of the two independently performed experiments showed an enhanced proliferation over a time course of 72 hours (Figure 3B). Hence, additional independent experiments would be needed to draw a final conclusion.

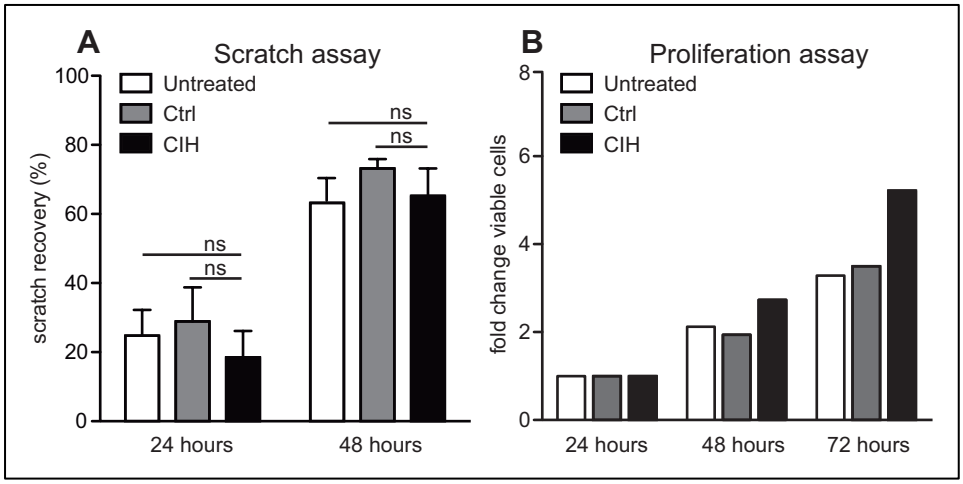

Figure 3: Effect of chronic intermittent hypoxia (CIH) on migratory and proliferative potential of T47D cells. (A) Scratch assay to compare migratory potential of CIH-conditioned cells (CIH) with their respective normoxic control cells (Ctrl) and with cells that were only cultured for a short time period (Untreated) (n=3). ns, not significant; one-way ANOVA followed by Tukey's Multiple Comparison Test. **(B)** Proliferation of CIH-conditioned cells (CIH), their respective normoxic control cells (Ctrl) and cells that were cultured for a short time period (Untreated) (n=2).

DISCUSSION

Here, we analyzed the effect of CIH on gene expression and cancer cell malignant properties in the estrogen receptor-positive breast cancer line T47D. T47D cells were cultured in CIH conditions and gene array analysis revealed changes in gene expression following CIH. Validation of the results in three independent experiments confirmed an upregulated expression of STC1 following CIH. A high expression of STC1 has been linked to a poor prognostic outcome and to the promotion of tumor progression (Liu et al., 2010; McCudden et al., 2004). Further, we could confirm a downregulated expression of SULF1. A low expression of SULF1 was found in different tumor entities and has been linked tumor progression (Abiatari et al., 2006; Lai et al., 2003; Lai et al., 2004). These findings indicate that CIH is an important regulator of cancer progression, which is mediated by the upregulation of oncogenes and/or downregulation of tumor suppressor genes. Upon identifying changes in gene expression, which suggested a translation into a more aggressive phenotype of T47D cells following CIH, different *in vitro* assays were performed. Analyzing the migratory potential, we could not observe any changes following CIH. Performing proliferation assays, suggested a trend towards an enhanced proliferation following CIH, but as only one of the two performed experiments showed an enhanced proliferation, additional independent experiments, as well as different *in vitro* and *in vivo* assays

would be needed in order to fully investigate the role of CIH on cancer cell malignant properties.

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6. Conclusions and future perspectives

Cancer is one of the biggest threats to our society with most of cancer-related deaths resulting from metastatic cancers. Breast cancer is the most common cancer in women worldwide and the second most common cancer overall (Ferlay et al., 2015). Much progress has been made in the fight against cancer, which includes an earlier diagnosis and better therapies. Still, many improvements are needed in the development of targeted therapies and especially in the treatment of metastatic disease.

In this thesis, we analyzed the role of chronic intermittent hypoxia (CIH) on breast cancer cell gene expression and malignant properties. The human triple-negative inflammatory breast cancer (IBC) cell line SUM149PT and the estrogen receptor-positive breast cancer cell line T47D were exposed to intermittent hypoxia (24 h 0.2% O_2, 48 h 21% O_2) for two month.

In vitro assays were performed in order to identify CIH-mediated changes in the behavior of SUM149PT and T47D cells. In SUM149PT cells, we observed a trend towards a differential attachment behavior following CIH. In all further performed assays, we could not observe significant changes in the cancer cell behavior. This might have different reasons. For example, SUM149PT cells origin from a patient with IBC and IBC is the most aggressive form of breast cancer. Hence, SUM149PT cells host already very aggressive features and one could assume that CIH did not cause a major additive enhancement in aggressiveness, which could have been detected with the performed *in vitro* assays.

The effects of CIH on the transcriptomic profile were analyzed in order to get insights into the signaling pathways which might be involved in the repeatedly reported enhancement of tumor aggressiveness upon intermittent hypoxia.

Chronic intermittent hypoxia (CIH) caused changes in gene expression in both cell lines, yet the observed changes did not overlap. Triple negative and ER-positive breast cancers have particular and opposing characteristics. As basal gene expression differs completely between these two cell lines, it is not surprising that the observed changes in gene expression were also different. Further, the number of differentially expressed genes was much higher in SUM149PT cells than in T47D cells. In SUM149PT cells, 531 protein-coding genes were more than 2-fold upregulated, while 379 genes were more than 2-fold downregulated. In T47D cells,

the number of differentially expressed genes was 10 times lower. In T47D cells, we positively validated the CIH-mediated differential expression of two extracellular matrix (ECM) proteins, which have been linked to tumor progression. Further analyses focused on SUM149PT cells, as inflammatory breast cancers have a much worse prognosis than ER-positive ones and hence further understanding of the disease is vital in order to develop better therapies. In SUM149PT cells, CIH caused a strong upregulation of pro-metastatic genes encoding ECM proteins and proteins involved in inflammatory processes. We identified an upregulated expression of matrix remodeling proteases, like the matrix metalloproteinases MMP2 and MMP9, and further ECM components like tenascin-C (TNC), versican, fibrillin, nidogen and collagen type VI. This suggests that CIH promotes changes in gene expression which may result in ECM remodeling, supporting tumor progression, particularly invasion and metastasis formation. We further confirmed an upregulated expression of tenascin-C upon intermittent hypoxia on mRNA and protein level. High levels of TNC have been linked to poor prognosis (Yoshida, Ishihara, Hirokawa, Kusakabe, & Sakakura, 1995), local and distant recurrence in breast carcinomas (Jahkola et al., 1998; Minn et al., 2005) and metastasis formation (Midwood, Hussenet, Langlois, & Orend, 2011; Orend, 2005; Saupe et al., 2013).

As TNC is a key factor in tumor progression, we focused on analyzing the signaling pathways that cause the elevated expression of TNC mediated by CIH. The oscillating changes in tissue oxygen availability during CIH are a source of oxidative stress due to reactive oxygen species (ROS) generation (Dewhirst, Cao, & Moeller, 2008). Hence, we decided to study the role of oxidative stress in TNC regulation. In fact, we could identify for the first time an oxidative stress mediated regulation of TNC in inflammatory breast cancer cells, which was dependent on activation of the NF-κB but not of other redox signaling pathways. Reports linking oxidative stress and TNC regulation are very rare. The only study showing a direct link between oxidative stress (H_2O_2 stimulation) and TNC regulation was performed in rat cardiac myocytes (Yamamoto et al., 1999). A few other studies postulated an indirect link between oxidative stress and TNC regulation (Aziz et al., 1997; Eba et al., 2013; Zou et al., 2013). However, to our knowledge there is no study reporting an oxidative stress mediated TNC regulation in breast cancer cells. Further, we could support an NF-κB dependent TNC regulation in inflammatory breast cancer cells by treating SUM149PT cells with Il-1β. Il-1β stimulation caused a strong induction of TNC protein

levels. Various reports exist showing TNC inducibility upon stimulation with IL-1α, IL-1β, TNF-α or LPS (Goh, Piccinini, Krausgruber, Udalova, & Midwood, 2010; Luo et al., 2015; Maqbool et al., 2013; Sarközi et al., 2015). However, none of the studies was performed in breast cancer cells.

Tumor inflammation has also been associated with cancer progression and metastasis formation and we observed an upregulated expression of proteins involved in inflammatory processes following CIH. Hanahan and Weinberg added the tumor-promoting inflammation as a new "enabling characteristic" to their original description of the hallmarks of cancer. This tumor-promoting inflammation contributes to the acquisition of other hallmarks by supplying bioactive molecules to the tumor microenvironment, including growth factors, pro-angiogenic factors and matrix-modifying enzymes (Hanahan & Weinberg, 2011).
TNC has been shown to activate toll-like receptor 4, followed by NF-κB activation and induction of pro-inflammatory cytokines and of TNC itself. TNC has been identified to drive the persistent joint inflammation observed in rheumatoid arthritis (Midwood et al., 2009). Further, TNC has been shown to drive the persistence of organ fibrosis in systemic sclerosis via toll-like receptor 4 (Bhattacharyya et al., 2016). Hence, dysregulated TNC may promote an autocrine loop leading to chronic inflammation in autoimmune diseases as well as in cancer.
In endothelial cells, IH has been shown to amplify a tumor-promoting pro-inflammatory phenotype with increased expression of inflammatory cytokines mediated via NF-κB activation (Tellier et al., 2015). CIH might also play an important role in IBC. Several studies indicated NF-κB to be constitutively activated in IBC (Lerebours et al., 2008; Van Laere et al., 2006). This constitutive NF-κB activation might be partly mediated through CIH and TNC.

TNC is now becoming increasingly used as a diagnostic and prognostic marker for a number of different cancers (Ide et al., 2007; Ishihara, Yoshida, Tamaki, & Sakakura, 1995; Tanaka et al., 2000). Also, it is the target of novel anti-cancer therapies. Several monoclonal antibodies have been developed that recognize tumor-specific TNC isoforms, hence targeting TNC in cancer but not in healthy tissue (Brack, Silacci, Birchler, & Neri, 2006). Some of these therapeutic antibodies have been examined in preclinical and clinical trials. One of these antibodies is the F16 antibody

that recognizes the A1 domain of TNC. Coupling the F16 antibody to interleukin-2 (IL-2) targets IL-2 to the tumor where it attracts the immune system to eliminate the tumor cells. Preclinical studies revealed reasonable effects, especially in combination with cytotoxic drugs (Mårlind et al., 2008), and suggested applicability to human cancer patients. Indeed, first clinical trials have been performed (Catania et al., 2015). Other attempts for the targeted delivery of radioisotopes or chemical agents to tumors include the generation of TNC specific aptamers (Daniels, Chen, Hicke, Swiderek, & Gold, 2003; Hicke et al., 2001). Further, a vaccination approach targeting several tumor specific antigens, including TNC domain C, is currently under investigation (Saupe et al., 2015). The development of TNC targeting therapies emphasizes the importance of TNC in cancer progression and hence in the treatment of cancer. Further understanding of its regulation on the transcriptional level might help to design strategies to prevent its induction during tumorigenesis.

This thesis identifies CIH in mediating tumor promotive gene expression changes. It further emphasizes the importance of CIH and NF-κB in TNC regulation and also in the regulation of other pro-metastatic genes in an IBC cells line. It reveals a putative novel mechanism involved in inflammatory breast cancer aggressiveness.

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7. Contributions to publications

Publication mentioned in this thesis

1. Gutsche, K., Blank, V., Fink, D., Wenger, R. H., Leo, C., and Scholz, C. C. (2016). Intermittent hypoxia confers pro-metastatic gene expression selectively through NF-κB in inflammatory breast cancer cells. **Submitted to Free Radical Biology and Medicine**.

All figures

Publications not mentioned in this thesis

1. Fuady, J. H., **Gutsche, K.**, Santambrogio, S., Varga, Z., Hoogewijs, D., and Wenger, R. H. (2016). Estrogen-dependent downregulation of hypoxia-inducible factor (HIF)-2α in invasive breast cancer cells. **Oncotarget,** in press.

Analysis of tissue microarrays for Figure 4A-C and Supplementary Figure S2.

2. Samartzis, E. P., **Gutsche, K.**, Dedes, K. J., Fink, D., Stucki, M., and Imesch, P. (2014)**.** Loss of ARID1A expression sensitizes cancer cells to PI3K- and AKT-inhibition. **Oncotarget,** 5(14), 5295-303.

Figures 2A, 2B, 2D, 2E, 4B, 4C

3. Larsen, D. H., Hari, F., Clapperton, J. A., Gwerder, M., **Gutsche, K.**, Altmeyer, M., Jungmichel, S., Toledo, L. I., Fink, D., Rask, M-B., Grofte, M., Lukas, C., Nielsen, M. L., Smerdon, S. J., Lukas, J., and Stucki, M. (2014). The NBS1-Treacle complex controls ribosomal RNA transcription in response to DNA damage. **Nature Cell Biology,** 16(8), 792-803.

ChIP experiments for Figure 2C-D

8. Curriculum Vitae

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07/2010 - 03/2011	**Graduate research assistant**, German Sport University Cologne, Institute of Cardiology and Sports Medicine
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IT Skills	MS-Office, Internet, Image J, Photoshop, Adobe Illustrator, GraphPad Prism, Corel Draw

Publications

Gutsche K, Blank V, Fink D, Wenger RH, Leo C, Scholz CC (2016) Intermittent hypoxia confers pro-metastatic gene expression selectively through NF-κB in inflammatory breast cancer cells. Submitted to Free Radical Biology and Medicine.

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Gehlert S, Weber S, Weidmann B, **Gutsche K**, Platen P, Graf C, Kappes-Horn K, Bloch W (2011) Cycling exercise-induced myofiber transitions in skeletal muscle depend on basal fiber type distribution. European Journal of Applied Physiology, 112(7), 2393-402.

Conferences and poster presentation

08/2015	11th ZIHP symposium; Zurich, Switzerland
05/2015	Keystone symposia meeting; Dublin, Ireland Hypoxia: From basic mechanisms to therapeutics
02/2014	Cancer Biology Student Retreat; Filzbach, Switzerland
06/2013	Jahreskongress der schweizerischen Gesellschaft für Gynäkologie und Geburtshilfe (SGGG); Lugano, Switzerland
04/2013	Cancer Research Retreat; Grindelwald, Switzerland
04/2013	12th Day of Clinical Research, Zurich, Switzerland

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